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TITLE: Tumor Antigens BFA4 and BCY1 for Prevention and / or Treatment of Cancer

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RELATED APPLICATIONS

This application claims priority to U.S. Ser. Nos. 60/394,346 filed July 3, 2002; 60/394,503 filed July 9, 2002; 60/411,833 filed September 18, 2002; and, 60/445,342 filed February 6, 2003, all of which being hereby incorporated by reference.

FIELD OF THE INVENTION

The present invention relates to a nucleic acid encoding a polypeptide and the use of the nucleic acid or polypeptide in preventing and / or treating cancer. In particular, the invention relates to improved vectors for the insertion and expression of foreign genes encoding tumor antigens for use in immunotherapeutic treatment of cancer.

BACKGROUND OF THE INVENTION

There has been tremendous increase in last few years in the development of cancer vaccines with Tumour-associated antigens (TAAs) due to the great advances in identification of molecules based on the expression profiling on primary tumours and normal cells with the help of several techniques such as high density microarray, SEREX, immunohistochemistry (IHC), RT-PCR, in-situ hybridization (ISH) and laser capture microscopy (Rosenberg, Immunity, 1999; Sgroi et al, 1999, Schena et al, 1995, Offringa et al, 2000). The TAAs are antigens expressed or over-expressed by tumour cells and could be specific to one or several tumours for example CEA antigen is expressed in colorectal, breast and lung cancers. Sgroi et al (1999) identified several genes differentially expressed in invasive and metastatic carcinoma cells with combined use of laser capture microdissection and cDNA microarrays. Several delivery systems like DNA or viruses could be used for therapeutic vaccination against human cancers (Bonnet et al, 2000) and can elicit immune responses and also break immune tolerance against TAAs. Tumour cells can be rendered more immunogenic by inserting transgenes encoding T cell co-stimulatory molecules such as B7.1 or cytokines such as IFN-γ, IL2, or GM-CSF, among others. Co-expression of a TAA and a cytokine or a co-stimulatory molecule can develop effective therapeutic vaccine (Hodge et al, 95, Bronte et al, 1995, Chamberlain et al, 1996).

There is a need in the art for reagents and methodologies useful in stimulating an immune response to prevent or treat cancers. The present invention provides such reagents and methodologies which overcome many of the difficulties encountered by others in attempting to treat cancer.

SUMMARY OF THE INVENTION

The present invention provides an immunogenic target for administration to a patient to prevent and / or treat cancer. In particular, the immunogenic target is a tumor antigen ("TA") and / or an angiogenesis-associated antigen ("AA"). In one embodiment, the immunogenic target is encoded by SEQ ID NO.: 1 or 3 or has the amino acid sequence of SEQ ID NO.: 2 or 4. In certain embodiments, the TA and / or AA are administered to a patient as a nucleic acid contained within a plasmid or other delivery vector, such as a recombinant virus. The TA and / or AA may also be administered in combination with an immune stimulator, such as a co-stimulatory molecule or adjuvant.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. BFA4 cDNA sequence.

Figure 2. BFA4 amino acid sequence.

Figure 3. BCY1 nucleotide (A) and amino acid (B) sequences.

DETAILED DESCRIPTION

The present invention provides reagents and methodologies useful for treating and / or preventing cancer. All references cited within this application are incorporated by reference.

In one embodiment, the present invention relates to the induction or enhancement of an immune response against one or more tumor antigens ("TA") to prevent and / or treat cancer. In certain embodiments, one or more TAs may be combined. In preferred embodiments, the immune response results from expression of a TA in a host cell following administration of a nucleic acid vector encoding the tumor antigen or the tumor antigen itself in the form of a peptide or polypeptide, for example.

As used herein, an "antigen" is a molecule (such as a polypeptide) or a portion thereof that produces an immune response in a host to whom the antigen has been administered. The

immune response may include the production of antibodies that bind to at least one epitope of the antigen and / or the generation of a cellular immune response against cells expressing an epitope of the antigen. The response may be an enhancement of a current immune response by, for example, causing increased antibody production, production of antibodies with increased affinity for the antigen, or an increased cellular response (i.e., increased T cells). An antigen that produces an immune response may alternatively be referred to as being immunogenic or as an immunogen. In describing the present invention, a TA may be referred to as an "immunogenic target".

TA includes both tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs), where a cancerous cell is the source of the antigen. A TAA is an antigen that is expressed on the surface of a tumor cell in higher amounts than is observed on normal cells or an antigen that is expressed on normal cells during fetal development. A TSA is an antigen that is unique to tumor cells and is not expressed on normal cells. TA further includes TAAs or TSAs, antigenic fragments thereof, and modified versions that retain their antigenicity.

TAs are typically classified into five categories according to their expression pattern, function, or genetic origin: cancer-testis (CT) antigens (i.e., MAGE, NY-ESO-1); melanocyte differentiation antigens (i.e., Melan A/MART-1, tyrosinase, gp100); mutational antigens (i.e., MUM-1, p53, CDK-4); overexpressed 'self' antigens (i.e., HER-2/neu, p53); and, viral antigens (i.e., HPV, EBV). For the purposes of practicing the present invention, a suitable TA is any TA that induces or enhances an anti-tumor immune response in a host to whom the TA has been administered. Suitable TAs include, for example, gp100 (Cox et al., *Science*, 264:716-719 (1994)), MART-1/Melan A (Kawakami et al., *J. Exp. Med.*, 180:347-352 (1994)), gp75 (TRP-1) (Wang et al., *J. Exp. Med.*, 186:1131-1140 (1996)), tyrosinase (Wolfel et al., *Eur. J. Immunol.*, 24:759-764 (1994); WO 200175117; WO 200175016; WO 200175007), NY-ESO-1 (WO 98/14464; WO 99/18206), melanoma proteoglycan (Hellstrom et al., *J. Immunol.*, 130:1467-1472 (1983)), MAGE family antigens (i.e., MAGE-1, 2,3,4,6,12, 51; Van der Bruggen et al., *Science*, 254:1643-1647 (1991); U.S. Pat. Nos. 6,235,525; CN 1319611), BAGE family antigens (Boel et al., *Immunity*, 2:167-175 (1995)), GAGE family antigens (i.e., GAGE-1,2; Van den Eynde et al., *J. Exp. Med.*, 182:689-698 (1995); U.S. Pat. No. 6,013,765), RAGE family antigens (i.e., RAGE-1; Gaugler et at., *Immunogenetics*, 44:323-330 (1996); U.S. Pat. No. 5,939,526), N-acetylglucosaminyltransferase-V (Guilloux et at., *J. Exp. Med.*, 183:1173-1183 (1996)), p15

(Robbins et al., *J. Immunol.* 154:5944-5950 (1995)), ß-catenin (Robbins et al., *J. Exp. Med.*, 183:1185-1192 (1996)), MUM-1 (Coulie et al., *Proc. Natl. Acad. Sci.* USA, 92:7976-7980 (1995)), cyclin dependent kinase-4 (CDK4) (Wolfel et al., *Science*, 269:1281-1284 (1995)), p21-*ras* (Fossum et at., *Int. J. Cancer,* 56:40-45 (1994)), BCR-*abl* (Bocchia et al., *Blood*, 85:2680-2684 (1995)), p53 (Theobald et al., *Proc. Natl. Acad. Sci.* USA, 92:11993-11997 (1995)), p185 HER2/neu (erb-B1; Fisk et al., *J. Exp. Med.*, 181:2109-2117 (1995)), epidermal growth factor receptor (EGFR) (Harris et al., Breast Cancer Res. Treat, 29:1-2 (1994)), carcinoembryonic antigens (CEA) (Kwong et al., *J. Natl. Cancer Inst.*, 85:982-990 (1995) U.S. Pat. Nos. 5,756,103; 5,274,087; 5,571,710; 6,071,716; 5,698,530; 6,045,802; EP 263933; EP 346710; and, EP 784483); carcinoma-associated mutated mucins (i.e., MUC-1 gene products; Jerome et al., *J. Immunol.*, 151:1654-1662 (1993)); EBNA gene products of EBV (i.e., EBNA-1; Rickinson et al., *Cancer Surveys*, 13:53-80 (1992)); E7, E6 proteins of human papillomavirus (Ressing et al., *J. Immunol*, 154:5934-5943 (1995)); prostate specific antigen (PSA; Xue et al., *The Prostate*, 30:73-78 (1997)); prostate specific membrane antigen (PSMA; Israeli, et al., *Cancer Res.*, 54:1807-1811 (1994)); idiotypic epitopes or antigens, for example, immunoglobulin idiotypes or T cell receptor idiotypes (Chen et al., *J. Immunol.*, 153:4775-4787 (1994)); KSA (U.S. Patent No. 5,348,887), kinesin 2 (Dietz, et al. Biochem Biophys Res Commun 2000 Sep 7;275(3):731-8), HIP-55, TGFβ-1 anti-apoptotic factor (Toomey, et al. Br J Biomed Sci 2001;58(3):177-83), tumor protein D52 (Bryne J.A., et al., Genomics, 35:523-532 (1996)), H1FT, NY-BR-1 (WO 01/47959), NY-BR-62, NY-BR-75, NY-BR-85, NY-BR-87, NY-BR-96 (Scanlan, M. Serologic and Bioinformatic Approaches to the Identification of Human Tumor Antigens, in *Cancer Vaccines 2000*, Cancer Research Institute, New York, NY), BFA4 (SEQ ID NOS.: 26 and 27), or BCY1 (SEQ ID NOS.: 28 and 29), including "wild-type" (i.e., normally encoded by the genome, naturally-occurring), modified, and mutated versions as well as other fragments and derivatives thereof. Any of these TAs may be utilized alone or in combination with one another in a co-immunization protocol.

In certain cases, it may be beneficial to co-immunize patients with both TA and other antigens, such as angiogenesis-associated antigens ("AA"). An AA is an immunogenic molecule (i.e., peptide, polypeptide) associated with cells involved in the induction and / or continued development of blood vessels. For example, an AA may be expressed on an endothelial cell ("EC"), which is a primary structural component of blood vessels. Where the cancer is cancer, it

is preferred that that the AA be found within or near blood vessels that supply a tumor. Immunization of a patient against an AA preferably results in an anti-AA immune response whereby angiogenic processes that occur near or within tumors are prevented and / or inhibited.

Exemplary AAs include, for example, vascular endothelial growth factor (i.e., VEGF; Bernardini, et al. *J. Urol.*, 2001, 166(4): 1275-9; Starnes, et al. *J. Thorac. Cardiovasc. Surg.*, 2001, 122(3): 518-23; Dias, et al. *Blood*, 2002, 99: 2179-2184), the VEGF receptor (i.e., VEGF-R, flk-1/KDR; Starnes, et al. *J. Thorac. Cardiovasc. Surg.*, 2001, 122(3): 518-23), EPH receptors (i.e., EPHA2; Gerety, et al. 1999, *Cell*, 4: 403-414), epidermal growth factor receptor (i.e., EGFR; Ciardeillo, et al. Clin. Cancer Res., 2001, 7(10): 2958-70), basic fibroblast growth factor (i.e., bFGF; Davidson, et al. Clin. Exp. Metastasis 2000,18(6): 501-7; Poon, et al. Am J. Surg., 2001, 182(3):298-304), platelet-derived cell growth factor (i.e., PDGF-B), platelet-derived endothelial cell growth factor (PD-ECGF; Hong, et al. J. Mol. Med., 2001, 8(2):141-8), transforming growth factors (i.e., TGF-α; Hong, et al. J. Mol. Med., 2001, 8(2):141-8), endoglin (Balza, et al. *Int. J. Cancer*, 2001, 94: 579-585), Id proteins (Benezra, R. Trends Cardiovasc. Med., 2001, 11(6):237-41), proteases such as uPA, uPAR, and matrix metalloproteinases (MMP-2, MMP-9; Djonov, et al. J. Pathol., 2001, 195(2):147-55), nitric oxide synthase (Am. J. Ophthalmol., 2001, 132(4):551-6), aminopeptidase (Rouslhati, E. Nature Cancer, 2: 84-90, 2002), thrombospondins (i.e., TSP-1, TSP-2; Alvarez, et al. Gynecol. Oncol., 2001, 82(2):273-8; Seki, et al. Int. J. Oncol., 2001, 19(2):305-10), k-*ras* (Zhang, et al. Cancer Res., 2001, 61(16):6050-4), *Wnt* (Zhang, et al. Cancer Res., 2001, 61(16):6050-4), cyclin-dependent kinases (CDKs; Drug Resist. Updat. 2000, 3(2):83-88), microtubules (Timar, et al. 2001. *Path. Oncol. Res.*, 7(2): 85-94), heat shock proteins (i.e., HSP90 (Timar, *supra*)), heparin-binding factors (i.e., heparinase; Gohji, et al. Int. J. Cancer, 2001, 95(5):295-301), synthases (i.e., ATP synthase, thymidilate synthase), collagen receptors, integrins (i.e., αυβ3, αυβ5, α1β1, α2β1, α5β1), or surface proteolglycan NG2, among others, including "wild-type" (i.e., normally encoded by the genome, naturally-occurring), modified, mutated versions as well as other fragments and derivatives thereof. Any of these targets may be suitable in practicing the present invention, either alone or in combination with one another or with other agents.

In certain embodiments, a nucleic acid molecule encoding an immunogenic target is utilized. The nucleic acid molecule may comprise or consist of a nucleotide sequence encoding

one or more immunogenic targets, or fragments or derivatives thereof, such as that contained in a DNA insert in an ATCC Deposit. The term "nucleic acid sequence" or "nucleic acid molecule" refers to a DNA or RNA sequence. The term encompasses molecules formed from any of the known base analogs of DNA and RNA such as, but not limited to 4-acetylcytosine, 8-hydroxy-N6-methyladenosine, aziridinyl-cytosine, pseudoisocytosine, 5-(carboxyhydroxylmethyl) uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxy-methylaminomethyluracil, dihydrouracil, inosine, N6-iso-pentenyladenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethyl-guanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyamino-methyl-2-thiouracil, beta-D-mannosylqueosine, 5' -methoxycarbonyl-methyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, oxybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, and 2,6-diaminopurine, among others.

An isolated nucleic acid molecule is one that: (1) is separated from at least about 50 percent of proteins, lipids, carbohydrates, or other materials with which it is naturally found when total nucleic acid is isolated from the source cells; (2) is not be linked to all or a portion of a polynucleotide to which the nucleic acid molecule is linked in nature; (3) is operably linked to a polynucleotide which it is not linked to in nature; and / or, (4) does not occur in nature as part of a larger polynucleotide sequence. Preferably, the isolated nucleic acid molecule of the present invention is substantially free from any other contaminating nucleic acid molecule(s) or other contaminants that are found in its natural environment that would interfere with its use in polypeptide production or its therapeutic, diagnostic, prophylactic or research use. As used herein, the term "naturally occurring" or "native" or "naturally found" when used in connection with biological materials such as nucleic acid molecules, polypeptides, host cells, and the like, refers to materials which are found in nature and are not manipulated by man. Similarly, "non-naturally occurring" or "non-native" as used herein refers to a material that is not found in nature or that has been structurally modified or synthesized by man.

The identity of two or more nucleic acid or polypeptide molecules is determined by comparing the sequences. As known in the art, "identity" means the degree of sequence

relatedness between nucleic acid molecules or polypeptides as determined by the match between the units making up the molecules (i.e., nucleotides or amino acid residues). Identity measures the percent of identical matches between the smaller of two or more sequences with gap alignments (if any) addressed by a particular mathematical model or computer program (i.e., an algorithm). Identity between nucleic acid sequences may also be determined by the ability of the related sequence to hybridize to the nucleic acid sequence or isolated nucleic acid molecule. In defining such sequences, the term "highly stringent conditions" and "moderately stringent conditions" refer to procedures that permit hybridization of nucleic acid strands whose sequences are complementary, and to exclude hybridization of significantly mismatched nucleic acids. Examples of "highly stringent conditions" for hybridization and washing are 0.015 M sodium chloride, 0.0015 M sodium citrate at 65-68°C or 0.015 M sodium chloride, 0.0015 M sodium citrate, and 50% formamide at 42°C. (see, for example, Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual* (2nd ed., Cold Spring Harbor Laboratory, 1989); Anderson *et al.*, *Nucleic Acid Hybridisation: A Practical Approach* Ch. 4 (IRL Press Limited)). The term "moderately stringent conditions" refers to conditions under which a DNA duplex with a greater degree of base pair mismatching than could occur under "highly stringent conditions" is able to form. Exemplary moderately stringent conditions are 0.015 M sodium chloride, 0.0015 M sodium citrate at 50-65°C or 0.015 M sodium chloride, 0.0015 M sodium citrate, and 20% formamide at 37-50°C. By way of example, moderately stringent conditions of 50°C in 0.015 M sodium ion will allow about a 21% mismatch. During hybridization, other agents may be included in the hybridization and washing buffers for the purpose of reducing non-specific and/or background hybridization. Examples are 0.1% bovine serum albumin, 0.1% polyvinyl-pyrrolidone, 0.1% sodium pyrophosphate, 0.1% sodium dodecylsulfate, $NaDodSO_4$, (SDS), ficoll, Denhardt's solution, sonicated salmon sperm DNA (or another non-complementary DNA), and dextran sulfate, although other suitable agents can also be used. The concentration and types of these additives can be changed without substantially affecting the stringency of the hybridization conditions. Hybridization experiments are usually carried out at pH 6.8-7.4; however, at typical ionic strength conditions, the rate of hybridization is nearly independent of pH.

In preferred embodiments of the present invention, vectors are used to transfer a nucleic acid sequence encoding a polypeptide to a cell. A vector is any molecule used to transfer a

nucleic acid sequence to a host cell. In certain cases, an expression vector is utilized. An expression vector is a nucleic acid molecule that is suitable for transformation of a host cell and contains nucleic acid sequences that direct and / or control the expression of the transferred nucleic acid sequences. Expression includes, but is not limited to, processes such as transcription, translation, and splicing, if introns are present. Expression vectors typically comprise one or more flanking sequences operably linked to a heterologous nucleic acid sequence encoding a polypeptide. Flanking sequences may be homologous (i.e., from the same species and / or strain as the host cell), heterologous (i.e., from a species other than the host cell species or strain), hybrid (i.e., a combination of flanking sequences from more than one source), or synthetic, for example.

A flanking sequence is preferably capable of effecting the replication, transcription and / or translation of the coding sequence and is operably linked to a coding sequence. As used herein, the term operably linked refers to a linkage of polynucleotide elements in a functional relationship. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence. However, a flanking sequence need not necessarily be contiguous with the coding sequence, so long as it functions correctly. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence and the promoter sequence may still be considered operably linked to the coding sequence. Similarly, an enhancer sequence may be located upstream or downstream from the coding sequence and affect transcription of the sequence.

In certain embodiments, it is preferred that the flanking sequence is a trascriptional regulatory region that drives high-level gene expression in the target cell. The transcriptional regulatory region may comprise, for example, a promoter, enhancer, silencer, repressor element, or combinations thereof. The transcriptional regulatory region may be either constitutive, tissue-specific, cell-type specific (i.e., the region is drives higher levels of transcription in a one type of tissue or cell as compared to another), or regulatable (i.e., responsive to interaction with a compound such as tetracycline). The source of a transcriptional regulatory region may be any prokaryotic or eukaryotic organism, any vertebrate or invertebrate organism, or any plant, provided that the flanking sequence functions in a cell by causing transcription of a nucleic acid within that cell. A wide variety of transcriptional regulatory regions may be utilized in practicing the present invention.

Suitable transcriptional regulatory regions include the CMV promoter (i.e., the CMV-immediate early promoter); promoters from eukaryotic genes (i.e., the estrogen-inducible chicken ovalbumin gene, the interferon genes, the gluco-corticoid-inducible tyrosine aminotransferase gene, and the thymidine kinase gene); and the major early and late adenovirus gene promoters; the SV40 early promoter region (Bernoist and Chambon, 1981, *Nature* 290:304-10); the promoter contained in the 3' long terminal repeat (LTR) of Rous sarcoma virus (RSV) (Yamamoto, *et al.*, 1980, *Cell* 22:787-97); the herpes simplex virus thymidine kinase (HSV-TK) promoter (Wagner *et al.*, 1981, *Proc. Natl. Acad. Sci. U.S.A.* 78:1444-45); the regulatory sequences of the metallothionine gene (Brinster *et al.*, 1982, *Nature* 296:39-42); prokaryotic expression vectors such as the beta-lactamase promoter (Villa-Kamaroff *et al.*, 1978, *Proc. Natl. Acad. Sci. U.S.A.*, 75:3727-31); or the tac promoter (DeBoer *et al.*, 1983, *Proc. Natl. Acad. Sci. U.S.A.*, 80:21-25). Tissue- and / or cell-type specific transcriptional control regions include, for example, the elastase I gene control region which is active in pancreatic acinar cells (Swift *et al.*, 1984, *Cell* 38:639-46; Ornitz *et al.*, 1986, *Cold Spring Harbor Symp. Quant. Biol.* 50:399-409 (1986); MacDonald, 1987, *Hepatology* 7:425-515); the insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, *Nature* 315:115-22); the immunoglobulin gene control region which is active in lymphoid cells (Grosschedl *et al.*, 1984, *Cell* 38:647-58; Adames *et al.*, 1985, *Nature* 318:533-38; Alexander *et al.*, 1987, *Mol. Cell. Biol.*, 7:1436-44); the mouse mammary tumor virus control region in testicular, breast, lymphoid and mast cells (Leder *et al.*, 1986, *Cell* 45:485-95); the albumin gene control region in liver (Pinkert *et al.*, 1987, *Genes and Devel.* 1:268-76); the alpha-feto-protein gene control region in liver (Krumlauf *et al.*, 1985, *Mol. Cell. Biol.*, 5:1639-48; Hammer *et al.*, 1987, *Science* 235:53-58); the alpha 1-antitrypsin gene control region in liver (Kelsey *et al.*, 1987, *Genes and Devel.* 1:161-71); the beta-globin gene control region in myeloid cells (Mogram *et al.*, 1985, *Nature* 315:338-40; Kollias *et al.*, 1986, *Cell* 46:89-94); the myelin basic protein gene control region in oligodendrocyte cells in the brain (Readhead *et al.*, 1987, *Cell* 48:703-12); the myosin light chain-2 gene control region in skeletal muscle (Sani, 1985, *Nature* 314:283-86); the gonadotropic releasing hormone gene control region in the hypothalamus (Mason *et al.*, 1986, *Science* 234:1372-78), and the tyrosinase promoter in melanoma cells (Hart, I. Semin Oncol 1996 Feb;23(1):154-8; Siders, et al. Cancer Gene Ther 1998 Sep-Oct;5(5):281-91), among others. Inducible promoters that are activated in the presence of a certain compound or condition such as light, heat, radiation,

tetracycline, or heat shock proteins, for example, may also be utilized (see, for example, WO 00/10612). Other suitable promoters are known in the art.

As described above, enhancers may also be suitable flanking sequences. Enhancers are cis-acting elements of DNA, usually about 10-300 bp in length, that act on the promoter to increase transcription. Enhancers are typically orientation- and position-independent, having been identified both 5' and 3' to controlled coding sequences. Several enhancer sequences available from mammalian genes are known (i.e., globin, elastase, albumin, alpha-feto-protein and insulin). Similarly, the SV40 enhancer, the cytomegalovirus early promoter enhancer, the polyoma enhancer, and adenovirus enhancers are useful with eukaryotic promoter sequences. While an enhancer may be spliced into the vector at a position 5' or 3' to nucleic acid coding sequence, it is typically located at a site 5' from the promoter. Other suitable enhancers are known in the art, and would be applicable to the present invention.

While preparing reagents of the present invention, cells may need to be transfected or transformed. Transfection refers to the uptake of foreign or exogenous DNA by a cell, and a cell has been transfected when the exogenous DNA has been introduced inside the cell membrane. A number of transfection techniques are well known in the art (i.e., Graham *et al.*, 1973, *Virology* 52:456; Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* (Cold Spring Harbor Laboratories, 1989); Davis *et al.*, *Basic Methods in Molecular Biology* (Elsevier, 1986); and Chu *et al.*, 1981, *Gene* 13:197). Such techniques can be used to introduce one or more exogenous DNA moieties into suitable host cells.

In certain embodiments, it is preferred that transfection of a cell results in transformation of that cell. A cell is transformed when there is a change in a characteristic of the cell, being transformed when it has been modified to contain a new nucleic acid. Following transfection, the transfected nucleic acid may recombine with that of the cell by physically integrating into a chromosome of the cell, may be maintained transiently as an episomal element without being replicated, or may replicate independently as a plasmid. A cell is stably transformed when the nucleic acid is replicated with the division of the cell.

The present invention further provides isolated immunogenic targets in polypeptide form. A polypeptide is considered isolated where it: (1) has been separated from at least about 50 percent of polynucleotides, lipids, carbohydrates, or other materials with which it is naturally found when isolated from the source cell; (2) is not linked (by covalent or noncovalent

interaction) to all or a portion of a polypeptide to which the "isolated polypeptide" is linked in nature; (3) is operably linked (by covalent or noncovalent interaction) to a polypeptide with which it is not linked in nature; or, (4) does not occur in nature. Preferably, the isolated polypeptide is substantially free from any other contaminating polypeptides or other contaminants that are found in its natural environment that would interfere with its therapeutic, diagnostic, prophylactic or research use.

Immunogenic target polypeptides may be mature polypeptides, as defined herein, and may or may not have an amino terminal methionine residue, depending on the method by which they are prepared. Further contemplated are related polypeptides such as, for example, fragments, variants (i.e., allelic, splice), orthologs, homologues, and derivatives, for example, that possess at least one characteristic or activity (i.e., activity, antigenicity) of the immunogenic target. Also related are peptides, which refers to a series of contiguous amino acid residues having a sequence corresponding to at least a portion of the polypeptide from which its sequence is derived. In preferred embodiments, the peptide comprises about 5-10 amino acids, 10-15 amino acids, 15-20 amino acids, 20-30 amino acids, or 30-50 amino acids. In a more preferred embodiment, a peptide comprises 9-12 amino acids, suitable for presentation upon Class I MHC molecules, for example.

A fragment of a nucleic acid or polypeptide comprises a truncation of the sequence (i.e., nucleic acid or polypeptide) at the amino terminus (with or without a leader sequence) and / or the carboxy terminus. Fragments may also include variants (i.e., allelic, splice), orthologs, homologues, and other variants having one or more amino acid additions or substitutions or internal deletions as compared to the parental sequence. In preferred embodiments, truncations and/or deletions comprise about 10 amino acids, 20 amino acids, 30 amino acids, 40 amino acids, 50 amino acids, or more. The polypeptide fragments so produced will comprise about 10 amino acids, 25 amino acids, 30 amino acids, 40 amino acids, 50 amino acids, 60 amino acids, 70 amino acids, or more. Such polypeptide fragments may optionally comprise an amino terminal methionine residue. It will be appreciated that such fragments can be used, for example, to generate antibodies or cellular immune responses to immunogenic target polypeptides.

A variant is a sequence having one or more sequence substitutions, deletions, and/or additions as compared to the subject sequence. Variants may be naturally occurring or artificially constructed. Such variants may be prepared from the corresponding nucleic acid

molecules. In preferred embodiments, the variants have from 1 to 3, or from 1 to 5, or from 1 to 10, or from 1 to 15, or from 1 to 20, or from 1 to 25, or from 1 to 30, or from 1 to 40, or from 1 to 50, or more than 50 amino acid substitutions, insertions, additions and/or deletions.

An allelic variant is one of several possible naturally-occurring alternate forms of a gene occupying a given locus on a chromosome of an organism or a population of organisms. A splice variant is a polypeptide generated from one of several RNA transcript resulting from splicing of a primary transcript. An ortholog is a similar nucleic acid or polypeptide sequence from another species. For example, the mouse and human versions of an immunogenic target polypeptide may be considered orthologs of each other. A derivative of a sequence is one that is derived from a parental sequence those sequences having substitutions, additions, deletions, or chemically modified variants. Variants may also include fusion proteins, which refers to the fusion of one or more first sequences (such as a peptide) at the amino or carboxy terminus of at least one other sequence (such as a heterologous peptide).

"Similarity" is a concept related to identity, except that similarity refers to a measure of relatedness which includes both identical matches and conservative substitution matches. If two polypeptide sequences have, for example, 10/20 identical amino acids, and the remainder are all non-conservative substitutions, then the percent identity and similarity would both be 50%. If in the same example, there are five more positions where there are conservative substitutions, then the percent identity remains 50%, but the percent similarity would be 75% (15/20). Therefore, in cases where there are conservative substitutions, the percent similarity between two polypeptides will be higher than the percent identity between those two polypeptides.

Substitutions may be conservative, or non-conservative, or any combination thereof. Conservative amino acid modifications to the sequence of a polypeptide (and the corresponding modifications to the encoding nucleotides) may produce polypeptides having functional and chemical characteristics similar to those of a parental polypeptide. For example, a "conservative amino acid substitution" may involve a substitution of a native amino acid residue with a non-native residue such that there is little or no effect on the size, polarity, charge, hydrophobicity, or hydrophilicity of the amino acid residue at that position and, in particlar, does not result in decreased immunogenicity. Suitable conservative amino acid substitutions are shown in **Table I**.

Table I

Original Residues	Exemplary Substitutions	Preferred Substitutions
Ala	Val, Leu, Ile	Val
Arg	Lys, Gln, Asn	Lys
Asn	Gln	Gln
Asp	Glu	Glu
Cys	Ser, Ala	Ser
Gln	Asn	Asn
Glu	Asp	Asp
Gly	Pro, Ala	Ala
His	Asn, Gln, Lys, Arg	Arg
Ile	Leu, Val, Met, Ala, Phe, Norleucine	Leu
Leu	Norleucine, Ile, Val, Met, Ala, Phe	Ile
Lys	Arg, 1,4 Diamino-butyric Acid, Gln, Asn	Arg
Met	Leu, Phe, Ile	Leu
Phe	Leu, Val, Ile, Ala, Tyr	Leu
Pro	Ala	Gly
Ser	Thr, Ala, Cys	Thr
Thr	Ser	Ser
Trp	Tyr, Phe	Tyr
Tyr	Trp, Phe, Thr, Ser	Phe
Val	Ile, Met, Leu, Phe, Ala, Norleucine	Leu

A skilled artisan will be able to determine suitable variants of polypeptide using well-known techniques. For identifying suitable areas of the molecule that may be changed without destroying biological activity (i.e., MHC binding, immunogenicity), one skilled in the art may target areas not believed to be important for that activity. For example, when similar polypeptides with similar activities from the same species or from other species are known, one skilled in the art may compare the amino acid sequence of a polypeptide to such similar polypeptides. By performing such analyses, one can identify residues and portions of the molecules that are conserved among similar polypeptides. It will be appreciated that changes in areas of the molecule that are not conserved relative to such similar polypeptides would be less likely to adversely affect the biological activity and/or structure of a polypeptide. Similarly, the residues required for binding to MHC are known, and may be modified to improve binding. However, modifications resulting in decreased binding to MHC will not be appropriate in most situations. One skilled in the art would also know that, even in relatively conserved regions, one

may substitute chemically similar amino acids for the naturally occurring residues while retaining activity. Therefore, even areas that may be important for biological activity or for structure may be subject to conservative amino acid substitutions without destroying the biological activity or without adversely affecting the polypeptide structure.

Other preferred polypeptide variants include glycosylation variants wherein the number and/or type of glycosylation sites have been altered compared to the subject amino acid sequence. In one embodiment, polypeptide variants comprise a greater or a lesser number of N-linked glycosylation sites than the subject amino acid sequence. An N-linked glycosylation site is characterized by the sequence Asn-X-Ser or Asn-X-Thr, wherein the amino acid residue designated as X may be any amino acid residue except proline. The substitution of amino acid residues to create this sequence provides a potential new site for the addition of an N-linked carbohydrate chain. Alternatively, substitutions that eliminate this sequence will remove an existing N-linked carbohydrate chain. Also provided is a rearrangement of N-linked carbohydrate chains wherein one or more N-linked glycosylation sites (typically those that are naturally occurring) are eliminated and one or more new N-linked sites are created. To affect O-linked glycosylation of a polypeptide, one would modify serine and / or threonine residues.

Additional preferred variants include cysteine variants, wherein one or more cysteine residues are deleted or substituted with another amino acid (*e.g.*, serine) as compared to the subject amino acid sequence set. Cysteine variants are useful when polypeptides must be refolded into a biologically active conformation such as after the isolation of insoluble inclusion bodies. Cysteine variants generally have fewer cysteine residues than the native protein, and typically have an even number to minimize interactions resulting from unpaired cysteines.

In other embodiments, the isolated polypeptides of the current invention include fusion polypeptide segments that assist in purification of the polypeptides. Fusions can be made either at the amino terminus or at the carboxy terminus of the subject polypeptide variant thereof. Fusions may be direct with no linker or adapter molecule or may be through a linker or adapter molecule. A linker or adapter molecule may be one or more amino acid residues, typically from about 20 to about 50 amino acid residues. A linker or adapter molecule may also be designed with a cleavage site for a DNA restriction endonuclease or for a protease to allow for the separation of the fused moieties. It will be appreciated that once constructed, the fusion polypeptides can be derivatized according to the methods described herein. Suitable fusion

segments include, among others, metal binding domains (e.g., a poly-histidine segment), immunoglobulin binding domains (i.e., Protein A, Protein G, T cell, B cell, Fc receptor, or complement protein antibody-binding domains), sugar binding domains (e.g., a maltose binding domain), and/or a "tag" domain (i.e., at least a portion of α-galactosidase, a strep tag peptide, a T7 tag peptide, a FLAG peptide, or other domains that can be purified using compounds that bind to the domain, such as monoclonal antibodies). This tag is typically fused to the polypeptide upon expression of the polypeptide, and can serve as a means for affinity purification of the sequence of interest polypeptide from the host cell. Affinity purification can be accomplished, for example, by column chromatography using antibodies against the tag as an affinity matrix. Optionally, the tag can subsequently be removed from the purified sequence of interest polypeptide by various means such as using certain peptidases for cleavage. As described below, fusions may also be made between a TA and a co-stimulatory components such as the chemokines CXC10 (IP-10), CCL7 (MCP-3), or CCL5 (RANTES), for example.

A fusion motif may enhance transport of an immunogenic target to an MHC processing compartment, such as the endoplasmic reticulum. These sequences, referred to as tranduction or transcytosis sequences, include sequences derived from HIV tat (see Kim et al. 1997 J. Immunol. 159:1666), *Drosophila* antennapedia (see Schutze-Redelmeier et al. 1996 J. Immunol. 157:650), or human period-1 protein (hPER1; in particular, SRRHHCRSKAKRSRHH).

In addition, the polypeptide or variant thereof may be fused to a homologous polypeptide to form a homodimer or to a heterologous polypeptide to form a heterodimer. Heterologous peptides and polypeptides include, but are not limited to: an epitope to allow for the detection and/or isolation of a fusion polypeptide; a transmembrane receptor protein or a portion thereof, such as an extracellular domain or a transmembrane and intracellular domain; a ligand or a portion thereof which binds to a transmembrane receptor protein; an enzyme or portion thereof which is catalytically active; a polypeptide or peptide which promotes oligomerization, such as a leucine zipper domain; a polypeptide or peptide which increases stability, such as an immunoglobulin constant region; and a polypeptide which has a therapeutic activity different from the polypeptide or variant thereof.

In certain embodiments, it may be advantageous to combine a nucleic acid sequence encoding an immunogenic target, polypeptide, or derivative thereof with one or more co-stimulatory component(s) such as cell surface proteins, cytokines or chemokines in a

composition of the present invention. The co-stimulatory component may be included in the composition as a polypeptide or as a nucleic acid encoding the polypeptide, for example. Suitable co-stimulatory molecules include, for instance, polypeptides that bind members of the CD28 family (i.e., CD28, ICOS; Hutloff, et al. *Nature* 1999, 397: 263–265; Peach, et al. *J Exp Med* 1994, 180: 2049–2058) such as the CD28 binding polypeptides B7.1 (CD80; Schwartz, 1992; Chen et al, 1992; Ellis, et al. *J. Immunol.*, 156(8): 2700-9) and B7.2 (CD86; Ellis, et al. *J. Immunol.*, 156(8): 2700-9); polypeptides which bind members of the integrin family (i.e., LFA-1 (CD11a / CD18); Sedwick, et al. *J Immunol* 1999, 162: 1367–1375; Wülfing, et al. *Science* 1998, 282: 2266–2269; Lub, et al. *Immunol Today* 1995, 16: 479–483) including members of the ICAM family (i.e., ICAM-1, -2 or -3); polypeptides which bind CD2 family members (i.e., CD2, signalling lymphocyte activation molecule (CDw150 or "SLAM"; Aversa, et al. *J Immunol* 1997, 158: 4036–4044)) such as CD58 (LFA-3; CD2 ligand; Davis, et al. *Immunol Today* 1996, 17: 177–187) or SLAM ligands (Sayos, et al. *Nature* 1998, 395: 462–469); polypeptides which bind heat stable antigen (HSA or CD24; Zhou, et al. *Eur J Immunol* 1997, 27: 2524–2528); polypeptides which bind to members of the TNF receptor (TNFR) family (i.e., 4-1BB (CD137; Vinay, et al. *Semin Immunol* 1998, 10: 481–489), OX40 (CD134; Weinberg, et al. *Semin Immunol* 1998, 10: 471–480; Higgins, et al. *J Immunol* 1999, 162: 486–493), and CD27 (Lens, et al. *Semin Immunol* 1998, 10: 491–499)) such as 4-1BBL (4-1BB ligand; Vinay, et al. *Semin Immunol* 1998, 10: 481–48; DeBenedette, et al. *J Immunol* 1997, 158: 551–559), TNFR associated factor-1 (TRAF-1; 4-1BB ligand; Saoulli, et al. *J Exp Med* 1998, 187: 1849–1862, Arch, et al. *Mol Cell Biol* 1998, 18: 558–565), TRAF-2 (4-1BB and OX40 ligand; Saoulli, et al. *J Exp Med* 1998, 187: 1849–1862; Oshima, et al. *Int Immunol* 1998, 10: 517–526, Kawamata, et al. *J Biol Chem* 1998, 273: 5808–5814), TRAF-3 (4-1BB and OX40 ligand; Arch, et al. *Mol Cell Biol* 1998, 18: 558–565; Jang, et al. *Biochem Biophys Res Commun* 1998, 242: 613–620; Kawamata S, et al. *J Biol Chem* 1998, 273: 5808–5814), OX40L (OX40 ligand; Gramaglia, et al. *J Immunol* 1998, 161: 6510–6517), TRAF-5 (OX40 ligand; Arch, et al. *Mol Cell Biol* 1998, 18: 558–565; Kawamata, et al. *J Biol Chem* 1998, 273: 5808–5814), and CD70 (CD27 ligand; Couderc, et al. *Cancer Gene Ther.*, 5(3): 163-75). CD154 (CD40 ligand or "CD40L"; Gurunathan, et al. *J. Immunol.*, 1998, 161: 4563-4571; Sine, et al. *Hum. Gene Ther.*, 2001, 12: 1091-1102) may also be suitable.

One or more cytokines may also be suitable co-stimulatory components or "adjuvants", either as polypeptides or being encoded by nucleic acids contained within the compositions of the present invention (Parmiani, et al. Immunol Lett 2000 Sep 15; 74(1): 41-4; Berzofsky, et al. Nature Immunol. 1: 209-219). Suitable cytokines include, for example, interleukin-2 (IL-2) (Rosenberg, et al. *Nature Med.* 4: 321-327 (1998)), IL-4, IL-7, IL-12 (reviewed by Pardoll, 1992; Harries, et al. J. Gene Med. 2000 Jul-Aug;2(4):243-9; Rao, et al. *J. Immunol.* 156: 3357-3365 (1996)), IL-15 (Xin, et al. *Vaccine*, 17:858-866, 1999), IL-16 (Cruikshank, et al. J. Leuk Biol. 67(6): 757-66, 2000), IL-18 (*J. Cancer Res. Clin. Oncol.* 2001. 127(12): 718-726), GM-CSF (CSF (Disis, et al. *Blood*, 88: 202-210 (1996)), tumor necrosis factor-alpha (TNF-α), or interferons such as IFN-α or INF-γ. Other cytokines may also be suitable for practicing the present invention, as is known in the art.

Chemokines may also be utilized. For example, fusion proteins comprising CXCL10 (IP-10) and CCL7 (MCP-3) fused to a tumor self-antigen have been shown to induce anti-tumor immunity (Biragyn, et al. *Nature Biotech.* 1999, 17: 253-258). The chemokines CCL3 (MIP-1α) and CCL5 (RANTES) (Boyer, et al. *Vaccine*, 1999, 17 (Supp. 2): S53-S64) may also be of use in practicing the present invention. Other suitable chemokines are known in the art.

It is also known in the art that suppressive or negative regulatory immune mechanisms may be blocked, resulting in enhanced immune responses. For instance, treatment with anti-CTLA-4 (Shrikant, et al. *Immunity*, 1996, 14: 145-155; Sutmuller, et al. *J. Exp. Med.*, 2001, 194: 823-832), anti-CD25 (Sutmuller, *supra*), anti-CD4 (Matsui, et al. *J. Immunol.*, 1999, 163: 184-193), the fusion protein IL13Ra2-Fc (Terabe, et al. *Nature Immunol.*, 2000, 1: 515-520), and combinations thereof (i.e., anti-CTLA-4 and anti-CD25, Sutmuller, *supra*) have been shown to upregulate anti-tumor immune responses and would be suitable in practicing the present invention.

Any of these components may be used alone or in combination with other agents. For instance, it has been shown that a combination of CD80, ICAM-1 and LFA-3 ("TRICOM") may potentiate anti-cancer immune responses (Hodge, et al. *Cancer Res.* 59: 5800-5807 (1999). Other effective combinations include, for example, IL-12 + GM-CSF (Ahlers, et al. *J. Immunol.*, 158: 3947-3958 (1997); Iwasaki, et al. *J. Immunol.* 158: 4591-4601 (1997)), IL-12 + GM-CSF + TNF-α (Ahlers, et al. *Int. Immunol.* 13: 897-908 (2001)), CD80 + IL-12 (Fruend, et al. *Int. J.*

Cancer, 85: 508-517 (2000); Rao, et al. *supra*), and CD86 + GM-CSF + IL-12 (Iwasaki, supra). One of skill in the art would be aware of additional combinations useful in carrying out the present invention.In addition, the skilled artisan would be aware of additional reagents or methods that may be used to modulate such mechanisms. These reagents and methods, as well as others known by those of skill in the art, may be utilized in practicing the present invention.

Additional strategies for improving the efficiency of nucleic acid-based immunization may also be used including, for example, the use of self-replicating viral replicons (Caley, et al. 1999. *Vaccine*, 17: 3124-2135; Dubensky, et al. 2000. *Mol. Med.* 6: 723-732; Leitner, et al. 2000. *Cancer Res.* 60: 51-55), codon optimization (Liu, et al. 2000. *Mol. Ther.*, 1: 497-500; Dubensky, *supra*; Huang, et al. 2001. *J. Virol.* 75: 4947-4951), *in vivo* electroporation (Widera, et al. 2000. *J. Immunol.* 164: 4635-3640), incorporation of CpG stimulatory motifs (Gurunathan, et al. *Ann. Rev. Immunol.*, 2000, 18: 927-974; Leitner, *supra*; Cho, et al. J. Immunol. 168(10):4907-13), sequences for targeting of the endocytic or ubiquitin-processing pathways (Thomson, et al. 1998. *J. Virol.* 72: 2246-2252; Velders, et al. 2001. *J. Immunol.* 166: 5366-5373), Marek's disease virus type 1 VP22 sequences (J. Virol. 76(6):2676-82, 2002), prime-boost regimens (Gurunathan, *supra*; Sullivan, et al. 2000. *Nature*, 408: 605-609; Hanke, et al. 1998. *Vaccine*, 16: 439-445; Amara, et al. 2001. *Science*, 292: 69-74), and the use of mucosal delivery vectors such as *Salmonella* (Darji, et al. 1997. *Cell*, 91: 765-775; Woo, et al. 2001. *Vaccine*, 19: 2945-2954). Other methods are known in the art, some of which are described below.

Chemotherapeutic agents, radiation, anti-angiogenic compounds, or other agents may also be utilized in treating and / or preventing cancer using immunogenic targets (Sebti, et al. Oncogene 2000 Dec 27;19(56):6566-73). For example, in treating metastatic breast cancer, useful chemotherapeutic agents include cyclophosphamide, doxorubicin, paclitaxel, docetaxel, navelbine, capecitabine, and mitomycin C, among others. Combination chemotherapeutic regimens have also proven effective including cyclophosphamide + methotrexate + 5-fluorouracil; cyclophosphamide + doxorubicin + 5-fluorouracil; or, cyclophosphamide + doxorubicin, for example. Other compounds such as prednisone, a taxane, navelbine, mitomycin C, or vinblastine have been utlized for various reasons. A majority of breast cancer patients have estrogen-receptor positive (ER+) tumors and in these patients, endocrine therapy (i.e., tamoxifen) is preferred over chemotherapy. For such patients, tamoxifen or, as a second line therapy,

progestins (medroxyprogesterone acetate or megestrol acetate) are preferred. Aromatase inhibitors (i.e., aminoglutethimide and analogs thereof such as letrozole) decrease the availability of estrogen needed to maintain tumor growth and may be used as second or third line endocrine therapy in certain patients.

Other cancers may require different chemotherapeutic regimens. For example, metastatic colorectal cancer is typically treated with Camptosar (irinotecan or CPT-11), 5-fluorouracil or leucovorin, alone or in combination with one another. Proteinase and integrin inhibitors such as as the MMP inhibitors marimastate (British Biotech), COL-3 (Collagenex), Neovastat (Aeterna), AG3340 (Agouron), BMS-275291 (Bristol Myers Squibb), CGS 27023A (Novartis) or the integrin inhibitors Vitaxin (Medimmune), or MED1522 (Merck KgaA) may also be suitable for use. As such, immunological targeting of immunogenic targets associated with colorectal cancer could be performed in combination with a treatment using those chemotherapeutic agents. Similarly, chemotherapeutic agents used to treat other types of cancers are well-known in the art and may be combined with the immunogenic targets described herein.

Many anti-angiogenic agents are known in the art and would be suitable for co-administration with the immunogenic target vaccines (see, for example, Timar, et al. 2001. *Pathology Oncol. Res.*, 7(2): 85-94). Such agents include, for example, physiological agents such as growth factors (i.e., ANG-2, NK1,2,4 (HGF), transforming growth factor beta (TGF-β)), cytokines (i.e., interferons such as IFN-α, -β, -γ, platelet factor 4 (PF-4), PR-39), proteases (i.e., cleaved AT-III, collagen XVIII fragment (Endostatin)), HmwKallikrein-d5 plasmin fragment (Angiostatin), prothrombin-F1-2, TSP-1), protease inhibitors (i.e., tissue inhibitor of metalloproteases such as TIMP-1, -2, or –3; maspin; plasminogen activator-inhibitors such as PAI-1; pigment epithelium derived factor (PEDF)), Tumstatin (available through ILEX, Inc.), antibody products (i.e., the collagen-binding antibodies HUIV26, HUI77, XL313; anti-VEGF; anti-integrin (i.e., Vitaxin, (Lxsys))), and glycosidases (i.e., heparinase-I, -III). "Chemical" or modified physiological agents known or believed to have anti-angiogenic potential include, for example, vinblastine, taxol, ketoconazole, thalidomide, dolestatin, combrestatin A, rapamycin (Guba, et al. 2002, *Nature Med.*, 8: 128-135), CEP-7055 (available from Cephalon, Inc.), flavone acetic acid, Bay 12-9566 (Bayer Corp.), AG3340 (Agouron, Inc.), CGS 27023A (Novartis), tetracylcine derivatives (i.e., COL-3 (Collagenix, Inc.)), Neovastat (Aeterna), BMS-

275291 (Bristol-Myers Squibb), low dose 5-FU, low dose methotrexate (MTX), irsofladine, radicicol, cyclosporine, captopril, celecoxib, D45152-sulphated polysaccharide, cationic protein (Protamine), cationic peptide-VEGF, Suramin (polysulphonated napthyl urea), compounds that interfere with the function or production of VEGF (i.e., SU5416 or SU6668 (Sugen), PTK787/ZK22584 (Novartis)), Distamycin A, Angiozyme (ribozyme), isoflavinoids, staurosporine derivatives, genistein, EMD121974 (Merck KcgaA), tyrphostins, isoquinolones, retinoic acid, carboxyamidotriazole, TNP-470, octreotide, 2-methoxyestradiol, aminosterols (i.e., squalamine), glutathione analogues (i.e., N-acteyl-L-cysteine), combretastatin A-4 (Oxigene), Eph receptor blocking agents (*Nature*, 414:933-938, 2001), Rh-Angiostatin, Rh-Endostatin (WO 01/93897), cyclic-RGD peptide, accutin-disintegrin, benzodiazepenes, humanized anti-avb3 Ab, Rh-PAI-2, amiloride, p-amidobenzamidine, anti-uPA ab, anti-uPAR Ab, L-phanylalanin-N-methylamides (i.e., Batimistat, Marimastat), AG3340, and minocycline. Many other suitable agents are known in the art and would suffice in practicing the present invention.

The present invention may also be utilized in combination with "non-traditional" methods of treating cancer. For example, it has recently been demonstrated that administration of certain anaerobic bacteria may assist in slowing tumor growth. In one study, *Clostridium novyi* was modified to eliminate a toxin gene carried on a phage episome and administered to mice with colorectal tumors (Dang, et al. *P.N.A.S. USA*, 98(26): 15155-15160, 2001). In combination with chemotherapy, the treatment was shown to cause tumor necrosis in the animals. The reagents and methodologies described in this application may be combined with such treatment methodologies.

Nucleic acids encoding immunogenic targets may be administered to patients by any of several available techniques. Various viral vectors that have been successfully utilized for introducing a nucleic acid to a host include retrovirus, adenovirus, adeno-associated virus (AAV), herpes virus, and poxvirus, among others. It is understood in the art that many such viral vectors are available in the art. The vectors of the present invention may be constructed using standard recombinant techniques widely available to one skilled in the art. Such techniques may be found in common molecular biology references such as *Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press), *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991. Academic Press,

San Diego, CA), and *PCR Protocols: A Guide to Methods and Applications* (Innis, et al. 1990. Academic Press, San Diego, CA).

Preferred retroviral vectors are derivatives of lentivirus as well as derivatives of murine or avian retroviruses. Examples of suitable retroviral vectors include, for example, Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), SIV, BIV, HIV and Rous Sarcoma Virus (RSV). A number of retroviral vectors can incorporate multiple exogenous nucleic acid sequences. As recombinant retroviruses are defective, they require assistance in order to produce infectious vector particles. This assistance can be provided by, for example, helper cell lines encoding retrovirus structural genes. Suitable helper cell lines include Ψ2, PA317 and PA12, among others. The vector virions produced using such cell lines may then be used to infect a tissue cell line, such as NIH 3T3 cells, to produce large quantities of chimeric retroviral virions. Retroviral vectors may be administered by traditional methods (i.e., injection) or by implantation of a "producer cell line" in proximity to the target cell population (Culver, K., *et al.*, 1994, *Hum. Gene Ther.*, 5 (3): 343-79; Culver, K., *et al.*, *Cold Spring Harb. Symp. Quant. Biol.*, 59: 685-90); Oldfield, E., 1993, *Hum. Gene Ther.*, 4 (1): 39-69). The producer cell line is engineered to produce a viral vector and releases viral particles in the vicinity of the target cell. A portion of the released viral particles contact the target cells and infect those cells, thus delivering a nucleic acid of the present invention to the target cell. Following infection of the target cell, expression of the nucleic acid of the vector occurs.

Adenoviral vectors have proven especially useful for gene transfer into eukaryotic cells (Rosenfeld, M., *et al.*, 1991, *Science*, 252 (5004): 431-4; Crystal, R., *et al.*, 1994, *Nat. Genet.*, 8 (1): 42-51), the study eukaryotic gene expression (Levrero, M., *et al.*, 1991, *Gene*, 101 (2): 195-202), vaccine development (Graham, F. and Prevec, L., 1992, *Biotechnology*, 20: 363-90), and in animal models (Stratford-Perricaudet, L., *et al.*, 1992, *Bone Marrow Transplant.*, 9 (Suppl. 1): 151-2 ; Rich, D., *et al.*, 1993, *Hum. Gene Ther.*, 4 (4): 461-76). Experimental routes for administrating recombinant Ad to different tissues *in vivo* have included intratracheal instillation (Rosenfeld, M., *et al.*, 1992, *Cell*, 68 (1): 143-55) injection into muscle (Quantin, B., *et al.*, 1992, *Proc. Natl. Acad. Sci. U.S.A.*, 89 (7): 2581-4), peripheral intravenous injection (Herz, J., and Gerard, R., 1993, *Proc. Natl. Acad. Sci. U.S.A.*, 90 (7): 2812-6) and stereotactic inoculation to brain (Le Gal La Salle, G., *et al.*, 1993, *Science*, 259 (5097): 988-90), among others.

Adeno-associated virus (AAV) demonstrates high-level infectivity, broad host range and specificity in integrating into the host cell genome (Hermonat, P., et al., 1984, *Proc. Natl. Acad. Sci. U.S.A.,* 81 (20): 6466-70). And Herpes Simplex Virus type-1 (HSV-1) is yet another attractive vector system, especially for use in the nervous system because of its neurotropic property (Geller, A., *et al.,* 1991, *Trends Neurosci.,* 14 (10): 428-32; Glorioso, *et al.*, 1995, *Mol. Biotechnol.,* 4 (1): 87-99; Glorioso, *et al.*, 1995, *Annu. Rev. Microbiol.,* 49: 675-710).

Poxvirus is another useful expression vector (Smith, et al. 1983, *Gene,* 25 (1): 21-8; Moss, et al, 1992, *Biotechnology,* 20: 345-62; Moss, et al, 1992, *Curr. Top. Microbiol. Immunol.*, 158: 25-38; Moss, et al. 1991. *Science*, 252: 1662-1667). Poxviruses shown to be useful include vaccinia, NYVAC, avipox, fowlpox, canarypox, ALVAC, and ALVAC(2), among others.

NYVAC (vP866) was derived from the Copenhagen vaccine strain of vaccinia virus by deleting six nonessential regions of the genome encoding known or potential virulence factors (see, for example, U.S. Pat. Nos. 5,364,773 and 5,494,807). The deletion loci were also engineered as recipient loci for the insertion of foreign genes. The deleted regions are: thymidine kinase gene (TK; J2R); hemorrhagic region (u; B13R+B14R); A type inclusion body region (ATI; A26L); hemagglutinin gene (HA; A56R); host range gene region (C7L-K1L); and, large subunit, ribonucleotide reductase (I4L). NYVAC is a genetically engineered vaccinia virus strain that was generated by the specific deletion of eighteen open reading frames encoding gene products associated with virulence and host range. NYVAC has been show to be useful for expressing TAs (see, for example, U.S. Pat. No. 6,265,189). NYVAC (vP866), vP994, vCP205, vCP1433, placZH6H4Lreverse, pMPC6H6K3E3 and pC3H6FHVB were also deposited with the ATCC under the terms of the Budapest Treaty, accession numbers VR-2559, VR-2558, VR-2557, VR-2556, ATCC-97913, ATCC-97912, and ATCC-97914, respectively.

ALVAC-based recombinant viruses (i.e., ALVAC-1 and ALVAC-2) are also suitable for use in practicing the present invention (see, for example, U.S. Pat. No. 5,756,103). ALVAC(2) is identical to ALVAC(1) except that ALVAC(2) genome comprises the vaccinia E3L and K3L genes under the control of vaccinia promoters (U.S. Pat. No. 6,130,066; Beattie et al., 1995a, 1995b, 1991; Chang et al., 1992; Davies et al., 1993). Both ALVAC(1) and ALVAC(2) have been demonstrated to be useful in expressing foreign DNA sequences, such as TAs (Tartaglia et al., 1993 a,b; U.S. Pat. No. 5,833,975). ALVAC was deposited under the terms of the Budapest

Treaty with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Va. 20110-2209, USA, ATCC accession number VR-2547.

Another useful poxvirus vector is TROVAC. TROVAC refers to an attenuated fowlpox that was a plaque-cloned isolate derived from the FP-1 vaccine strain of fowlpoxvirus which is licensed for vaccination of 1 day old chicks. TROVAC was likewise deposited under the terms of the Budapest Treaty with the ATCC, accession number 2553.

"Non-viral" plasmid vectors may also be suitable in practicing the present invention. Preferred plasmid vectors are compatible with bacterial, insect, and / or mammalian host cells. Such vectors include, for example, PCR-II, pCR3, and pcDNA3.1 (Invitrogen, San Diego, CA), pBSII (Stratagene, La Jolla, CA), pET15 (Novagen, Madison, WI), pGEX (Pharmacia Biotech, Piscataway, NJ), pEGFP-N2 (Clontech, Palo Alto, CA), pETL (BlueBacII, Invitrogen), pDSR-alpha (PCT pub. No. WO 90/14363) and pFastBacDual (Gibco-BRL, Grand Island, NY) as well as Bluescript® plasmid derivatives (a high copy number COLE1-based phagemid, Stratagene Cloning Systems, La Jolla, CA), PCR cloning plasmids designed for cloning Taq-amplified PCR products (*e.g.*, TOPO™ TA cloning® kit, PCR2.1® plasmid derivatives, Invitrogen, Carlsbad, CA). Bacterial vectors may also be used with the current invention. These vectors include, for example, *Shigella, Salmonella, Vibrio cholerae, Lactobacillus, Bacille calmette guérin (BCG), and Streptococcus* (see for example, WO 88/6626; WO 90/0594; WO 91/13157; WO 92/1796; and WO 92/21376). Many other non-viral plasmid expression vectors and systems are known in the art and could be used with the current invention.

Suitable nucleic acid delivery techniques include DNA-ligand complexes, adenovirus-ligand-DNA complexes, direct injection of DNA, $CaPO_4$ precipitation, gene gun techniques, electroporation, and colloidal dispersion systems, among others. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The preferred colloidal system of this invention is a liposome, which are artificial membrane vesicles useful as delivery vehicles *in vitro* and *in vivo*. RNA, DNA and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley, R., *et al.*, 1981, *Trends Biochem. Sci.*, 6: 77). The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in

combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations. Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingolipids, cerebrosides, and gangliosides. Particularly useful are diacylphosphatidylglycerols, where the lipid moiety contains from 14-18 carbon atoms, particularly from 16-18 carbon atoms, and is saturated. Illustrative phospholipids include egg phosphatidylcholine, dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine.

An immunogenic target may also be administered in combination with one or more adjuvants to boost the immune response. Exemplary adjuvants are shown in Table II below:

Table II

Types of Immunologic Adjuvants

Type of Adjuvant	General Examples	Specific Examples/References
Gel-type	Aluminum hydroxide/phosphate ("alum adjuvants")	(Aggerbeck and Heron, 1995)
	Calcium phosphate	(Relyveld, 1986)
Microbial	Muramyl dipeptide (MDP)	(Chedid et al., 1986)
	Bacterial exotoxins	Cholera toxin (CT), *E.coli* labile toxin (LT)(Freytag and Clements, 1999)
	Endotoxin-based adjuvants	Monophosphoryl lipid A (MPL) (Ulrich and Myers, 1995)
	Other bacterial	CpG oligonucleotides (Corral and Petray, 2000), BCG sequences (Krieg, et al. *Nature*, 374:576), tetanus toxoid (Rice, et al. *J. Immunol.* , 2001, 167: 1558-1565)
Particulate	Biodegradable Polymer microspheres	(Gupta et al., 1998)
	Immunostimulatory complexes (ISCOMs)	(Morein and Bengtsson, 1999)
	Liposomes	(Wassef et al., 1994)
Oil-emulsion and surfactant-based adjuvants	Freund's incomplete adjuvant	(Jensen et al., 1998)
	Microfluidized emulsions	MF59 (Ott et al., 1995)
		SAF (Allison and Byars, 1992) (Allison, 1999)
	Saponins	QS-21 (Kensil, 1996)
Synthetic	Muramyl peptide derivatives	Murabutide (Lederer, 1986) Threony-MDP (Allison, 1997)
	Nonionic block copolymers	L121 (Allison, 1999)
	Polyphosphazene (PCPP)	(Payne et al., 1995)

	Synthetic polynucleotides	Poly A:U, Poly I:C (Johnson, 1994)
	Thalidomide derivatives	CC-4047/ACTIMID (J. Immunol., 168(10):4914-9)

The immunogenic targets of the present invention may also be used to generate antibodies for use in screening assays or for immunotherapy. Other uses would be apparent to one of skill in the art. The term "antibody" includes antibody fragments, as are known in the art, including Fab, Fab_2, single chain antibodies (Fv for example), humanized antibodies, chimeric antibodies, human antibodies, produced by several methods as are known in the art. Methods of preparing and utilizing various types of antibodies are well-known to those of skill in the art and would be suitable in practicing the present invention (see, for example, Harlow, et al. *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988; Harlow, et al. *Using Antibodies: A Laboratory Manual, Portable Protocol No. 1*, 1998; Kohler and Milstein, Nature, 256:495 (1975)); Jones et al. Nature, 321:522-525 (1986); Riechmann et al. Nature, 332:323-329 (1988); Presta (Curr. Op. Struct. Biol., 2:593-596 (1992); Verhoeyen et al. (Science, 239:1534-1536 (1988); Hoogenboom et al., J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581 (1991); Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985); Boerner et al., J. Immunol., 147(1):86-95 (1991); Marks et al., Bio/Technology 10, 779-783 (1992); Lonberg et al., Nature 368 856-859 (1994); Morrison, Nature 368 812-13 (1994); Fishwild et al., Nature Biotechnology 14, 845-51 (1996); Neuberger, Nature Biotechnology 14, 826 (1996); Lonberg and Huszar, Intern. Rev. Immunol. 13 65-93 (1995); as well as U.S. Pat. Nos. 4,816,567; 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; and, 5,661,016). The antibodies or derivatives therefrom may also be conjugated to therapeutic moieties such as cytotoxic drugs or toxins, or active fragments thereof such as diptheria A chain, exotoxin A chain, ricin A chain, abrin A chain, curcin, crotin, phenomycin, enomycin, among others. Cytotoxic agents may also include radiochemicals. Antibodies and their derivatives may be incorporated into compositions of the invention for use *in vitro* or *in vivo*.

Nucleic acids, proteins, or derivatives thereof representing an immunogenic target may be used in assays to determine the presence of a disease state in a patient, to predict prognosis, or to determine the effectiveness of a chemotherapeutic or other treatment regimen. Expression profiles, performed as is known in the art, may be used to determine the relative level of expression of the immunogenic target. The level of expression may then be correlated with base

levels to determine whether a particular disease is present within the patient, the patient's prognosis, or whether a particular treatment regimen is effective. For example, if the patient is being treated with a particular chemotherapeutic regimen, an decreased level of expression of an immunogenic target in the patient's tissues (i.e., in peripheral blood) may indicate the regimen is decreasing the cancer load in that host. Similarly, if the level of expresssion is increasing, another therapeutic modality may need to be utilized. In one embodiment, nucleic acid probes corresponding to a nucleic acid encoding an immunogenic target may be attached to a biochip, as is known in the art, for the detection and quantification of expression in the host.

It is also possible to use nucleic acids, proteins, derivatives therefrom, or antibodies thereto as reagents in drug screening assays. The reagents may be used to ascertain the effect of a drug candidate on the expression of the immunogenic target in a cell line, or a cell or tissue of a patient. The expression profiling technique may be combined with high throughput screening techniques to allow rapid identification of useful compounds and monitor the effectiveness of treatment with a drug candidate (see, for example, Zlokarnik, et al., Science 279, 84-8 (1998)). Drug candidates may be chemical compounds, nucleic acids, proteins, antibodies, or derivatives therefrom, whether naturally occurring or synthetically derived. Drug candidates thus identified may be utilized, among other uses, as pharmaceutical compositions for administration to patients or for use in further screening assays.

Administration of a composition of the present invention to a host may be accomplished using any of a variety of techniques known to those of skill in the art. The composition(s) may be processed in accordance with conventional methods of pharmacy to produce medicinal agents for administration to patients, including humans and other mammals (i.e., a "pharmaceutical composition"). The pharmaceutical composition is preferably made in the form of a dosage unit containing a given amount of DNA, viral vector particles, polypeptide or peptide, for example. A suitable daily dose for a human or other mammal may vary widely depending on the condition of the patient and other factors, but, once again, can be determined using routine methods.

The pharmaceutical composition may be administered orally, parentally, by inhalation spray, rectally, intranodally, or topically in dosage unit formulations containing conventional pharmaceutically acceptable carriers, adjuvants, and vehicles. The term "pharmaceutically acceptable carrier" or "physiologically acceptable carrier" as used herein refers to one or more formulation materials suitable for accomplishing or enhancing the delivery of a nucleic acid,

polypeptide, or peptide as a pharmaceutical composition. A "pharmaceutical composition" is a composition comprising a therapeutically effective amount of a nucleic acid or polypeptide. The terms "effective amount" and "therapeutically effective amount" each refer to the amount of a nucleic acid or polypeptide used to induce or enhance an effective immune response. It is preferred that compositions of the present invention provide for the induction or enhancement of an anti-tumor immune response in a host which protects the host from the development of a tumor and / or allows the host to eliminate an existing tumor from the body.

For oral administration, the pharmaceutical composition may be of any of several forms including, for example, a capsule, a tablet, a suspension, or liquid, among others. Liquids may be administered by injection as a composition with suitable carriers including saline, dextrose, or water. The term parenteral as used herein includes subcutaneous, intravenous, intramuscular, intrasternal, infusion, or intraperitoneal administration. Suppositories for rectal administration of the drug can be prepared by mixing the drug with a suitable non-irritating excipient such as cocoa butter and polyethylene glycols that are solid at ordinary temperatures but liquid at the rectal temperature.

The dosage regimen for immunizing a host or otherwise treating a disorder or a disease with a composition of this invention is based on a variety of factors, including the type of disease, the age, weight, sex, medical condition of the patient, the severity of the condition, the route of administration, and the particular compound employed. For example, a poxviral vector may be administered as a composition comprising 1 x 10^6 infectious particles per dose. Thus, the dosage regimen may vary widely, but can be determined routinely using standard methods.

A prime-boost regimen may also be utilized (WO 01/30382 A1) in which the targeted immunogen is initially administered in a priming step in one form followed by a boosting step in which the targeted immunogen is administered in another form. The form of the targeted immunogen in the priming and boosting steps are different. For instance, if the priming step utilized a nucleic acid, the boost may be administered as a peptide. Similarly, where a priming step utilized one type of recombinant virus (i.e., ALVAC), the boost step may utilize another type of virus (i.e., NYVAC). This prime-boost method of administration has been shown to induce strong immunological responses.

While the compositions of the invention can be administered as the sole active pharmaceutical agent, they can also be used in combination with one or more other compositions

or agents (i.e., other immunogenic targets, co-stimulatory molecules, adjuvants). When administered as a combination, the individual components can be formulated as separate compositions administered at the same time or different times, or the components can be combined as a single composition.

Injectable preparations, such as sterile injectable aqueous or oleaginous suspensions, may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally acceptable diluent or solvent. Suitable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution, among others. For instance, a viral vector such as a poxvirus may be prepared in 0.4% NaCl. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed, including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

For topical administration, a suitable topical dose of a composition may be administered one to four, and preferably two or three times daily. The dose may also be administered with intervening days during which no does is applied. Suitable compositions may comprise from 0.001% to 10% w/w, for example, from 1% to 2% by weight of the formulation, although it may comprise as much as 10% w/w, but preferably not more than 5% w/w, and more preferably from 0.1% to 1% of the formulation. Formulations suitable for topical administration include liquid or semi-liquid preparations suitable for penetration through the skin (*e.g.*, liniments, lotions, ointments, creams, or pastes) and drops suitable for administration to the eye, ear, or nose.

The pharmaceutical compositions may also be prepared in a solid form (including granules, powders or suppositories). The pharmaceutical compositions may be subjected to conventional pharmaceutical operations such as sterilization and/or may contain conventional adjuvants, such as preservatives, stabilizers, wetting agents, emulsifiers, buffers etc. Solid dosage forms for oral administration may include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the active compound may be admixed with at least one inert diluent such as sucrose, lactose, or starch. Such dosage forms may also comprise, as in normal practice, additional substances other than inert diluents, *e.g.*, lubricating agents such as magnesium stearate. In the case of capsules, tablets, and pills, the dosage forms may also comprise buffering agents. Tablets and pills can additionally be prepared with enteric coatings. Liquid dosage

forms for oral administration may include pharmaceutically acceptable emulsions, solutions, suspensions, syrups, and elixirs containing inert diluents commonly used in the art, such as water. Such compositions may also comprise adjuvants, such as wetting sweetening, flavoring, and perfuming agents.

Pharmaceutical compositions comprising a nucleic acid or polypeptide of the present invention may take any of several forms and may be administered by any of several routes. In preferred embodiments, the compositions are administered via a parenteral route (intradermal, intramuscular or subcutaneous) to induce an immune response in the host. Alternatively, the composition may be administered directly into a lymph node (intranodal) or tumor mass (i.e., intratumoral administration). For example, the dose could be administered subcutaneously at days 0, 7, and 14. Suitable methods for immunization using compositions comprising TAs are known in the art, as shown for p53 (Hollstein et al., 1991), p21-ras (Almoguera et al., 1988), HER-2 (Fendly et al., 1990), the melanoma-associated antigens (MAGE-1; MAGE-2) (van der Bruggen et al., 1991), p97 (Hu et al., 1988), melanoma-associated antigen E (WO 99/30737) and carcinoembryonic antigen (CEA) (Kantor et al., 1993; Fishbein et al., 1992; Kaufman et al., 1991), among others.

Preferred embodiments of administratable compositions include, for example, nucleic acids or polypeptides in liquid preparations such as suspensions, syrups, or elixirs. Preferred injectable preparations include, for example, nucleic acids or polypeptides suitable for parental, subcutaneous, intradermal, intramuscular or intravenous administration such as sterile suspensions or emulsions. For example, a recombinant poxvirus may be in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose or the like. The composition may also be provided in lyophilized form for reconstituting, for instance, in isotonic aqueous, saline buffer. In addition, the compositions can be co-administered or sequentially administered with other antineoplastic, anti-tumor or anti-cancer agents and/or with agents which reduce or alleviate ill effects of antineoplastic, anti-tumor or anti-cancer agents.

A kit comprising a composition of the present invention is also provided. The kit can include a separate container containing a suitable carrier, diluent or excipient. The kit can also include an additional anti-cancer, anti-tumor or antineoplastic agent and/or an agent that reduces or alleviates ill effects of antineoplastic, anti-tumor or anti-cancer agents for co- or sequential-

administration. Additionally, the kit can include instructions for mixing or combining ingredients and/or administration.

A better understanding of the present invention and of its many advantages will be had from the following examples, given by way of illustration.

EXAMPLES

Example 1

BFA4 Tumor Antigen

The BFA4 sequence was found to be the "trichorhinophalangeal syndrome 1" (TRPS-1) gene (Genebank ID #6684533; Momeniet et al, Nature Genetics, 24(1), 71-74,2000), a known transcription factor with no function attributed previously in any form of cancer. The BFA4 cDNA sequence is shown in **Fig. 1** and the deduced amino acid sequence is shown in **Fig. 2**.

A. BFA4 Peptides and Polyclonal Antisera

For monitoring purposes, rabbit anti-BFA4 polyclonal antibodies were generated. Six peptides (22-mers) were designed and synthesized to elicit antibody response to BFA4, as shown below:

CLP 2589	MVRKKNPPLRNVASEGEGQILE	BFA4 (1-22)
CLP 2590	SPKATEETGQAQSGQANCQGLS	BFA4 (157-178)
CLP 2591	VAKPSEKNSNKSIPALQSSDSG	BFA4 (371-392)
CLP 2592	NHLQGSDGQQSVKESKEHSCTK	BFA4 (649-670)
CLP 2593	NGEQIIRRRTRKRLNPEALQAE	BFA4 (940-961)
CLP 2594	ANGASKEKTKAPPNVKNEGPLNV	BFA4 (1178-1199)

Rabbits were immunized with the peptides, serum was isolated, and the following antibody titers were observed:

Rabbit #	**Peptide**	Titer (Bleed 2)	Titer (Final Bleed)
1,2	CLP2589	800000, 1600000	2560000, 2560000
3,4	CLP2590	12800, 6400	40000, 40000
5,6	CLP2591	400000, 400000	320000, 320000
7,8	CLP2592	25600, 12800	80000, 40000
9,10	CLP2593	3200000, 51200	2560000, 160000
11,12	CLP2594	409600, 409600	320000, 320000

These peptides were also modified by coupling with KLH peptides to enhance immune responses as shown below:

BFA4(1-22)	KLH-MVRKKNPPLRNVASEGEGQILE	(CLP-2589)
BFA4(157-178)	KLH-SPKATEETGQAQSGQANCQGLS	(CLP-2590)
BFA4(371-392)	KLH-VAKPSEKNSNKSIPALQSSDSG	(CLP-2591)
BFA4(649-670)	KLH-NHLQGSDGQQSVKESKEHSCTK	(CLP-2592)
BFA4(940-961)	KLH-NGEQIIRRRTRKRLNPEALQAE	(CLP-2593)
BFA4(1178-1200)	KLH-ANGASKEKTKAPPNVKNEGPLNV	(CLP-2594)

The pcDNA3.2BFA4 (3.6 mg) was also used for DNA immunization to generate polyclonal sera in chickens.

B. Cloning of BFA4

Complete cDNA sequence for BFA4 is ~10kb and gene is expressed in BT474 ductal carcinoma cells. Primers 7717 (forward primer) and 7723 (reverse primer) were designed to amplify full-length BFA4 gene by amplification of 4kb, 7kb or 10kb products by RT-PCR.

Primer 7717: BFA4-BamH1/F1 (5' end forward) with Kozak:

5' CGGGATCCACCATGGTCCGGAAAAAGAACCCC 3'(BamHI for DNA3.1, MP76)

Primer 7723: BFA4-BamHI /R1 (3' end reverse 4kb):

5' CGGGATCCCTCTTTAGGTTTTCCATTTTTTTCCAC 3' (BamHI for DNA3.1, MP76)

Ten mg of total RNA isolated and frozen in different batches from BT-474 cells using Trizol as indicated by the manufacturer (Gibco BRL) was used in RT-PCR to amplify the BFA4 gene. RT-PCR conditions were optimized using Taq Platinum High Fidelity enzyme, OPC (Oligo Purification Cartridge; Applied Biosystems) purified primers and purified total RNA/polyA mRNA (BT 474 cells). Optimization resulted in a 4.0kb fragment as a single band.

To re-amplify the BFA4 sequence, mRNA was treated with DNase per manufacturers' instructions (Gibco BRL). The 4kb DNA was reamplified using PCR using primers 7717 and 7723 primers (10pmole/microlitre) and Taq Platinum High Fidelity polymerase (GIBCO BRL) enzyme. Thermocycler conditions for both sets of reactions were as under: 94°C (2 min),

followed by 30 cycles of 94°C (30 sec), 52°C (30sec), 67°C (4 min) and 67°C (5 min) and finally 40°C for 10 min. Three BFA4 clones were identified after pCR2.1/TOPO-TA cloning.

Several mutations were identified during analysis of the BFA4 sequence. To correct these sequences, the BamHI/XhoI fragment (5') of the BFA4 gene from clone JB-3552-1-2 (pCR2.1/TOPO/ BFA4) was exchanged with the XhoI/BamHI fragment (3') of the BFA4 gene from clone JB-3552-1-4 (pCR2.1/TOPO/BFA4). This recombined fragment was then ligated into pMCS5 BamHI/CAP. Clone JB-3624-1-5 was generated and found to contain the correct sequence.

Nucleotide 344 of the isolated BFA4 clone was different from the reported sequence (C in BFA4, T in TRPS-1). The change resulted in a phe to ser amino acid change. To change this sequence to the reported sequence, the EcoRI/BglII fragment (5') of the BFA4 gene from clone JB-3552-1-2 (pCR2.1/TOPO/BFA4) was subcloned into pUC8:2 to generate clone JB-3631-2. This clone was used as a template for Quickchange (Stratagene) mutagenesis to change amino acid 115 of the BFA4 protein from a serine to a phenylalanine as in the TRPS1 protein. The selected clone was JB-3648-2-3. Mutagenesis was also repeated with pMCS5 BFA4 (BT474) as a template for Quickchange (Stratagene) mutagenesis to change amino acid 115 of the BFA4 protein from a serine to a phenylalanine as in the TRPS1 protein. Several clones were found to be correct by DNA sequencing and one of the clones (JB-3685-1-18) was used for further subcloning.

JB-3685-1-18 was then used to subclone the BFA4 coding sequence into the *Bam*HI sites of four different expression vectors: 1) the poxviral (NYVAC) vector pSD554VC (COPAK/H6; JB-3707-1-7); 2) pcDNA3.1/Zeo (+) (JB-3707-3-2); 3) pCAMycHis (JB-3707-5-1); and, 4) Semiliki Forest virus alphaviral replicon vector pMP76 (JB-3735-1-23). The BFA4 coding sequence within JB-3707-1-7, JB-3707-5-1, and JB-3735-1-23 was confirmed by DNA sequencing.

A stop codon was introduced near the end of the cloned sequence in the pcDNA3.1/Zeo/BFA4 construct (JB-3707-3-2). A unique EcoR1 site was opened and filled in to introduce a stop codon in-frame with BFA4 coding sequence. Several putative clones were identified by the loss of EcoR1 site, however three clones (JB-3756-1-2; JB-3756-3-1; and JB-3756-4-1) were sequenced. All three were found to be correct in the area of the fill-in. Clone JB-3756-3-1 identified as having the correct sequence and orientation.

Myc and myc/his tags (Evans et al, 1985) were introduced using oligonucleotides, which were annealed and ligated into the pcDNA3.1/Zeo/BFA4 construct (JB-3707-3-2) at the EcoRI /EcoRV sites. Several clones were obtained for these constructs. Three clones having the correct sequences and orientations were obtained: 1) PcDNA3.1/Zeo/BFA4/myc-tag (JB-3773-1-2); 2) PcDNA3.1/Zeo/BFA4/mychis-tag (JB-3773-2-1); and, 3) PcDNA3.1/Zeo/BFA4/mychis-tag (JB-3773-2-2).

C. Expression of BFA4

1. Expression from poxviral vectors

The pSD554VC (COPAK/H6; JB-3707-1-7) vector was used to generate NYVAC-BFA4 virus. *In vitro* recombination was performed with plasmid COPAK/H6/BFA4 and NYVAC in RK13/CEF cells. NYVAC-BFA4 (vP2033-NYVAC-RK13) was generated and amplified to P3 level after completion of three enrichments with final stock concentrations of 1.12 x 10^9/ml (10ml). Vero cells were infected with NYVAC-BFA4 at an M.O.I. of 0.5 pfu/cell. Lysates and media were harvested 24h post-infection to confirm expression of BFA4 protein. One-twentieth of the concentrated media and 1/40 of the lysate were loaded onto a western blot and incubated with rabbit antisera against the BFA4 peptides CLP 2589, 2591, 2598 and 2594 (see above for peptide sequences and preparation of anti-BFA4 antisera). An approximate 120kD band was detected in both the lysate and the concentrated media of NYVAC-BFA4-infected Vero cells which was not evident in either Vero control cells ("mock-infected"), Vero cells infected with the parental NYVAC virus, or concentrated media.

2. Expression from pcDNA3.1-based vectors

Transient transfection studies were performed to verify expression of BFA4 from the pcDNA-based vectors and to analyze quality of polyclonal sera raised against BFA4 peptides. The following constructs were used to study expression of BFA4 gene: pcDNA 3.1 zeo^R/BFA4, pMP76/BFA4, pcDNA 3.1 zeo^R/ BFA4/Myc tag and pcDNA 3.1 zeo^R/ BFA4/MycHis tag. BFA4 expression plasmids (5μg and 10 μg) were co-transfected with pGL3 Luciferase (1□g) (Promega) with the Gene porter reagent (Gene Therapy Systems) as the transfection reagent. At 48h post-transfection, whole cell extract was prepared by scraping cells in cell lysis reagent

(200µl) and 1 cycle of freeze-thaw (–20°C freeze, 37°C thaw). Transfection efficiency was quantitated by analyzing expression of the luciferase reporter gene by measuring Relative Luciferase Units (RLU) in duplicate. Similar RLU values were obtained in the samples co-transfected with luciferase construct in the presence and absence of BFA4 expression vectors. There was no significant difference observed in toxicity or RLU values with differential amount (5µg and 10 µg) of BFA4 expression vectors. Preliminary western blot analysis using alkaline phosphatase system with the CHOK1 cell extracts (pCDNA3.1 /zeo/ BFA4/MycHisTag) and an anti-BFA4 polyclonal antisera, revealed a band at approximately 120kDa band in extracts of BFA4 vector-transfected cells.

A stable transfection study was initiated to obtain stable clones of BFA4 expressing COS A2 cells. These cells are useful for *in vitro* stimulation assays. pcDNA 3.1 zeo^R/BFA4 (2.5µg and 20 µg), and pcDNA 3.1 zeo ^{R}/ BFA4/MycHis tag (2.5µg) were used to study expression of BFA4). pGL3 Luciferase (2.5µg) was used as a control vector to monitor transfection efficiency. The Gene porter reagent was used to facilitate transfection of DNA vectors. After 48h post-transfection, whole cell extract were prepared by scraping cells in the cell lysis reagent (200µl) and 1 cycle of freeze-thaw at –20°C/37°C for first experiment. Transfected cells obtained from the second experiment were trypsinized, frozen stock established and cells were plated in increasing concentrations of Zeocin (0, 250, 500, 750 and 1000µg/ml). Non-transfected CosA2cells survived at 60-80 % confluency for three weeks at 100µg/ml (Zeocin) and 10% confluency at 250µg/ml (Zeocin). However, after three weeks, at higher drug concentration (500-1000µg/ml), live cells were not observed in the plates containing non-transfected cells and high Zeocin concentration (500-1000µg/ml).

Several Zeocin-resistant clones growing in differential drug concentrations (Zeocin-250, 500, 750 and 1000µg/ml) were picked from 10 cm plates after three weeks. These clones were further expanded in a 3.5 cm plate(s) in the presence of Zeocin at 500, 750 and 1000 µg/ml. Frozen lots of these clones were prepared and several clones from each pool (pcDNA 3.1 zeo^R/BFA4, and pcDNA 3.1 zeo^R/ BFA4/MycHis tag) were expanded to T75 cm^2 flasks in the presence of Zeocin at 1mg/ml. Five clones from each pool (pcDNA 3.1 zeo^R/BFA4, and pcDNA 3.1 zeo^R/ BFA4/MycHis tag) were expanded to T75 cm^2 flasks in the presence of Zeocin at 1mg/ml. Cells are maintained under Zeocin drug (1mg/ml) selection. Six clones were used in

BFA4 peptide-pulsed target experiment, and two clones were found to express BFA4 at a moderate level by immunological assays. The non-adherent cell lines K562A2 and EL4A2 were also transfected with these vectors to generate stable cell lines.

3. Prokaryotic expression vector

The BamHI –Xho-1 fragment (1.5 Kbp) fragment encoding N-terminal 54kDa BFDA4 from pCDNA3.1/BFA4 was cloned into pGEX4T1-6His (Veritas) plasmid. This vector contains the tac promoter followed by the N-terminal glutathione S-transferase (GST~26kDa) and a hexahistidine tag to C terminus of the GST fusion protein.

The BFA4-N54 expression plasmid was transformed into BL21 cells and grown at 25°C in antibiotic selection medium (2L culture) to an OD (600nm) and thereafter induced with 1mM IPTG. GST-BFA4-N54 was found to be soluble protein. Clarified extract of the soluble fraction was adsorbed batchwise to glutathione-Sepharose 4B and eluted with 10mM reduced glutathione. Fractions were analyzed after estimation of protein concentration and TCA precipitation. Specific polypeptide of Mr=85kDa in the eluate was confirmed by SDS-PAGE. The recombinant protein was purified by gluathione-Sepharose was absorbed on a NiNTA column for further purification. The bound protein was eluted with 0.25M imidazole. The protein was dialyzed versus TBS containing 40% Glycerol, resulting in 4.5 mg GST-BFA4-N54-6 His (N terminus BFA4 protein) protein. Expression of BFA4 was confirmed using the rabbit anti-BFA4 polyclonal antibody by western blot.

D. Therapeutic anti-BFA4 immune responses

1. BFA4 peptides

In addition to genetic immunization vectors for BFA4, immunological reagents for BFA4 have been generated. A library of 100 nonamer peptides spanning the BFA4 gene product was synthesized. The peptides were chosen based on their potential ability to bind to HLA-A*0201. **Table V** lists 100 nonamer peptide epitopes for HLA-A*0201 from the BFA4 protein tested (see below):

PEPTIDE DESIGNATION	SEQUENCE	POSITION IN PROTEIN
CLP- 2421	MVRKKNPPL	BFA4 (1-9)ı˜
CLP- 2422	KKNPPLRNV	BFA4 (4-12)ı˜
CLP- 2423	VASEGEGQI	BFA4 (12-20)ı˜
CLP- 2424	QILEPIGTE	BFA4 (19-27)ı˜
CLP- 2425	RNMLAFSFP	BFA4 (108-116)ı˜
CLP- 2426	NMLAFSFPA	BFA4 (109-117)ı˜
CLP- 2427	MLAFSFPAA	BFA4 (110-118)ı˜
CLP- 2428	FSFPAAGGV	BFA4 (113-121)ı˜
CLP- 2429	AAGGVCEPL	BFA4 (117-125)ı˜
CLP- 2430	SGQANCQGL	BFA4 (170-178)ı˜
CLP- 2431	ANCQGLSPV	BFA4 (172-180)ı˜
CLP- 2432	GLSPVSVAS	BFA4 (176-184)ı˜
CLP- 2433	SVASKNPQV	BFA4 (181-189)ı˜
CLP- 2434	RLNKSKTDL	BFA4 (196-204)ı˜
CLP- 2435	NDNPDPAPL	BFA4 (207-215)ı˜
CLP- 2436	DPAPLSPEL	BFA4 (211-219)ı˜
CLP- 2437	ELQDFKCNI	BFA4 (218-216)ı˜
CLP- 2438	GLHNRTRQD	BFA4 (249-257)ı˜
CLP- 2439	ELDSKILAL	BFA4 (259-267)ı˜
CLP- 2440	KILALHNMV	BFA4 (263-271)ı˜
CLP- 2441	ALHNMVQFS	BFA4 (266-284)ı˜
CLP- 2442	VNRSVFSGV	BFA4 (282-290)ı˜
CLP- 2443	FSGVLQDIN	BFA4 (287-295)ı˜
CLP- 2444	DINSSRPVL	BFA4 (293-301)ı˜
CLP- 2445	VLLNGTYDV	BFA4 (300-308)ı˜
CLP- 2446	FCNFTYMGN	BFA4 (337-345)ı˜
CLP- 2447	YMGNSSTEL	BFA4 (342-350)ı˜
CLP- 2448	FLQTHPNKI	BFA4 (354-362)ı˜
CLP- 2449	KASLPSSEV	BFA4 (363-371)ı˜
CLP- 2450	DLGKWQDKI	BFA4 (393-401)ı˜
CLP- 2451	VKAGDDTPV	BFA4 (403-411)ı˜
CLP- 2452	FSCESSSSL	BFA4 (441-449)ı˜
CLP- 2453	KLLEHYGKQ	BFA4 (450-458)ı˜
CLP- 2454	GLNPELNDK	BFA4 (466-474)ı˜
CLP- 2455	GSVINQNDL	BFA4 (478-486)ı˜
CLP- 2456	SVINQNDLA	BFA4 (479-487)ı˜
CLP- 2457	FCDFRYSKS	BFA4 (527-535)ı˜
CLP- 2458	SHGPDVIVV	BFA4 (535-543)ı˜
CLP- 2459	PLLRHYQQL	BFA4 (545-553)ı˜
CLP- 2460	GLCSPEKHL	BFA4 (570-578)ı˜
CLP- 2461	HLGEITYPF	BFA4 (577-585)ı˜
CLP- 2462	LGEITYPFA	BFA4 (578-586)ı˜
CLP- 2463	HCALLLLHL	BFA4 (594-602)ı˜
CLP- 2464	ALLLLHLSP	BFA4 (596-604)ı˜
CLP- 2465	LLLLHLSPG	BFA4 (597-605)ı˜
CLP- 2466	LLLHLSPGA	BFA4 (598-606)ı˜
CLP- 2467	LLHLSPGAA	BFA4 (599-607)ı˜
CLP- 2468	FTTPDVDVL	BFA4 (621-629)ı˜
CLP- 2469	TTPDVDVLL	BFA4 (622-630)ı˜
CLP- 2470	VLLFHYESV	BFA4 (628-636)ı˜
CLP- 2471	FITQVEEEI	BFA4 (673-681)ı˜
CLP- 2472	FTAADTQSL	BFA4 (699-707)ı˜
CLP- 2473	SLLEHFNTV	BFA4 (706-714)ı˜

PEPTIDE DESIGNATION	SEQUENCE	POSITION IN PROTEIN
CLP- 2474	STIKEEPKI	BFA4 (734-742)ı"
CLP- 2475	KIDFRVYNL	BFA4 (741-749)ı"
CLP- 2476	NLLTPDSKM	BFA4 (748-756)ı"
CLP- 2479	VTWRGADIL	BFA4 (792-800)ı"
CLP- 2480	ILRGSPSYT	BFA4 (799-807)ı"
CLP- 2481	YTQASLGLL	BFA4 (806-814)ı"
CLP- 2482	ASLGLLTPV	BFA4 (809-817)ı"
CLP- 2483	GLLTPVSGT	BFA4 (812-820)ı"
CLP- 2484	GTQEQTKTL	BFA4 (819-827)ı"
CLP- 2485	KTLRDSPNV	BFA4 (825-833)ı"
CLP- 2486	HLARPIYGL	BFA4 (837-845)ı"
CLP- 2487	PIYGLAVET	BFA4 (841-849)ı"
CLP- 2488	LAVETKGFL	BFA4 (845-853)ı"
CLP- 2489	FLQGAPAGG	BFA4 (852-860)ı"
CLP- 2490	AGGEKSGAL	BFA4 (858-866)ı"
CLP- 2491	GALPQQYPA	BFA4 (864-872)ı"
CLP- 2492	ALPQQYPAS	BFA4 (865-873)ı"
CLP- 2493	FCANCLTTK	BFA4 (895-903)ı"
CLP- 2494	ANGGYVCNA	BFA4 (911-919)ı"
CLP- 2495	NACGLYQKL	BFA4 (918-926)ı"
CLP- 2496	GLYQKLHST	BFA4 (921-929)ı"
CLP- 2497	KLHSTPRPL	BFA4 (925-933)ı"
CLP- 2498	STPRPLNII	BFA4 (928-936)ı"
CLP- 2499	RLNPEALQA	BFA4 (952-960)ı"
CLP- 2500	VLVSQTLDI	BFA4 (1020-1028)ı"
CLP- 2501	DIHKRMQPL	BFA4 (1027-1035)ı"
CLP- 2502	RMQPLHIQI	BFA4 (1031-1039)ı"
CLP- 2503	YPLFGLPFV	BFA4 (1092-1100)ı"
CLP- 2504	GLPFVHNDF	BFA4 (1096-1104)ı"
CLP- 2505	FVHNDFQSE	BFA4 (1099-1107)ı"
CLP- 2506	SVPGNPHYL	BFA4 (1120-1128)ı"
CLP- 2507	GNPHYLSHV	BFA4 (1123-1131)ı"
CLP- 2508	HYLSHVPGL	BFA4 (1126-1134)ı"
CLP- 2509	YVPYPTFNL	BFA4 (1141-1149)ı"
CLP- 2510	FNLPPHFSA	BFA4 (1147-1155)ı"
CLP- 2511	NLPPHFSAV	BFA4 (1148-1156)ı"
CLP- 2512	SAVGSDNDI	BFA4 (1154-1162)ı"
CLP- 2513	KNEGPLNVV	BFA4 (1192-1200)ı"
CLP- 2514	TKCVHCGIV	BFA4 (1215-1223)ı"
CLP- 2515	CVHCGIVFL	BFA4 (1217-1225)ı"
CLP- 2516	CGIVFLDEV	BFA4 (1220-1228)ı"
CLP- 2517	FLDEVMYAL	BFA4 (1224-1232)ı"
CLP- 2518	VMYALHMSC	BFA4 (1228-1236)ı"
CLP- 2519	FQCSICQHL	BFA4 (1243-1251)ı"
CLP- 2520	GLHRNNAQV	BFA4 (1265-1273)

The peptide library was pooled into separate groups containing 7-10 different peptides for immunological testing as shown in **Table VI** (see below). In addition to a peptide library spanning BFA4, a recombinant protein spanning the N-terminal 300 amino acids (positions 1-300) has been synthesized and purified from *E. coli*.

PEPTIDE GROUP	PEPTIDE NUMBER	SEQUENCE
1	CLP- 2421	MVRKKNPPL
	CLP- 2422	KKNPPLRNV
	CLP- 2423	VASEGEGQI
	CLP- 2424	QILEPIGTE
	CLP- 2425	RNMLAFSFP
	CLP- 2426	NMLAFSFPA
	CLP- 2427	MLAFSFPAA
	CLP- 2428	FSFPAAGGV
	CLP- 2429	AAGGVCEPL
	CLP- 2430	SGQANCQGL
2	CLP- 2431	ANCQGLSPV
	CLP- 2432	GLSPVSVAS
	CLP- 2433	SVASKNPQV
	CLP- 2434	RLNKSKTDL
	CLP- 2435	NDNPDPAPL
	CLP- 2436	DPAPLSPEL
	CLP- 2437	ELQDFKCNI
	CLP- 2438	GLHNRTRQD
	CLP- 2439	ELDSKILAL
	CLP- 2440	KILALHNMV
3	CLP- 2441	ALHNMVQFS
	CLP- 2442	VNRSVFSGV
	CLP- 2443	FSGVLQDIN
	CLP- 2444	DINSSRPVL
	CLP- 2445	VLLNGTYDV
	CLP- 2446	FCNFTYMGN
	CLP- 2447	YMGNSSTEL
	CLP- 2448	FLQTHPNKI
	CLP- 2449	KASLPSSEV
	CLP- 2450	DLGKWQDKI
4	CLP- 2451	VKAGDDTPV
	CLP- 2452	FSCESSSSL
	CLP- 2453	KLLEHYGKQ
	CLP- 2454	GLNPELNDK
	CLP- 2455	GSVINQNDL
	CLP- 2456	SVINQNDLA
	CLP- 2457	FCDFRYSKS
	CLP- 2458	SHGPDVIVV
	CLP- 2459	PLLRHYQQL
	CLP- 2460	GLCSPEKHL
5	CLP- 2461	HLGEITYPF
	CLP- 2462	LGEITYPFA
	CLP- 2463	HCALLLLHL
	CLP- 2464	ALLLLHLSP
	CLP- 2465	LLLLHLSPG
	CLP- 2466	LLLHLSPGA
	CLP- 2467	LLHLSPGAA
	CLP- 2468	FTTPDVDVL
	CLP- 2469	TTPDVDVLL
	CLP- 2470	VLLFHYESV

PEPTIDE GROUP	PEPTIDE NUMBER	SEQUENCE
6	CLP- 2471	FITQVEEEI
	CLP- 2472	FTAADTQSL
	CLP- 2473	SLLEHFNTV
	CLP- 2474	STIKEEPKI
	CLP- 2475	KIDFRVYNL
	CLP- 2476	NLLTPDSKM
	CLP- 2477	KMGEPVSES
	CLP- 2478	GLKEKVWTE
	CLP- 2479	VTWRGADIL
	CLP- 2480	ILRGSPSYT
7	CLP- 2481	YTQASLGLL
	CLP- 2482	ASLGLLTPV
	CLP- 2483	GLLTPVSGT
	CLP- 2484	GTQEQTKTL
	CLP- 2485	KTLRDSPNV
	CLP- 2486	HLARPIYGL
	CLP- 2487	PIYGLAVET
	CLP- 2488	LAVETKGFL
	CLP- 2489	FLQGAPAGG
	CLP- 2490	AGGEKSGAL
8	CLP- 2491	GALPQQYPA
	CLP- 2492	ALPQQYPAS
	CLP- 2493	FCANCLTTK
	CLP- 2494	ANGGYVCNA
	CLP- 2495	NACGLYQKL
	CLP- 2496	GLYQKLHST
	CLP- 2497	KLHSTPRPL
	CLP- 2498	STPRPLNII
	CLP- 2499	RLNPEALQA
	CLP- 2500	VLVSQTLDI
9	CLP- 2501	DIHKRMQPL
	CLP- 2502	RMQPLHIQI
	CLP- 2503	YPLFGLPFV
	CLP- 2504	GLPFVHNDF
	CLP- 2505	FVHNDFQSE
	CLP- 2506	SVPGNPHYL
	CLP- 2507	GNPHYLSHV
	CLP- 2508	HYLSHVPGL
	CLP- 2509	YVPYPTFNL
	CLP- 2510	FNLPPHFSA
10	CLP- 2511	NLPPHFSAV
	CLP- 2512	SAVGSDNDI
	CLP- 2513	KNEGPLNVV
	CLP- 2514	TKCVHCGIV
	CLP- 2515	CVHCGIVFL
	CLP- 2516	CGIVFLDEV
	CLP- 2517	FLDEVMYAL
	CLP- 2518	VMYALHMSC
	CLP- 2519	FQCSICQHL
	CLP- 2520	GLHRNNAQV

2. Immune reactivity of BFA4 peptides and generation of human effector T cells:

The BFA4 peptides were grouped into different pools of 7-10 peptides for immunological testing. Dissolved peptide pools were pulsed onto autologous HLA-A*0201 dendritic cells and used to activate autologous T-cell-enriched PBMC preparations. Activated T cells from each peptide-pool-stimulated culture were re-stimulated another 3 to 5 times using CD40L-activated autologous B-cells. IFN-γ ELISPOT analysis and assays for CTL killing of peptide-pulsed target cells was performed to demonstrate the immunogenicity of these epitopes from BFA4.

Human T cells demonstrated effector cell activity against a number of pools of peptides from the BFA4 protein, as shown by their ability to secrete IFN-γ in ELISPOT assays. These experiments were repeated after different rounds of APC stimulation resulting in the same reactive peptide groups. Peptide groups 1, 2, 4, 5, 6, 7, 8, 9, and 10 were found to be immunoreactive in these assays. Subsequently, these reactive peptide groups were de-convoluted in additional IFN-γ ELISPOT assays in which single peptides from each group were tested separately. The individual peptides from BFA4 peptide groups 1, 5 6, 7, 8, 9, and 10 in ELISPOT assays. This analysis revealed a number of individual strongly reactive peptides from the BFA4 protein recognized by human T cells. It was also observed that many of these single peptides also induced CTL activity killing peptide-loaded human T2 lymphoma cell targets. These peptides are listed in **Table VII**:

Table VII

List of highly immunoreactive peptides from BFA4

Strong IFN-γ Killing		Strong CTL Killing	
CLP 2425	**RNMLAFSFP**	CLP 2425	**RNMLAFSFP**
CLP 2426	**NMLAFSFPA**	CLP 2426	**NMLAFSFPA**
CLP 2427	**MLAFSFPAA**	CLP 2427	**MLAFSFPAA**
CLP 2461	**HLGEITYPF**		
CLP 2468	**FTTPDVDVL**	CLP 2468	**FTTPDVDVL**
CLP 2470	**VLLFHYESV**	CLP 2470	**VLLFHYYESV**
CLP 2474	**KIDFRVYNL**		
CLP 2482	**ASLGLLTPV**	CLP 2482	**ASLGLLTPV**
CLP 2486	**HLARPIYGL**	CLP 2486	**HLARPIYGL**
CLP 2495	**NACGLYQKL**	CLP 2495	**NACGLYQKL**
CLP 2497	**KLHSTPRPL**		
CLP 2499	**RLNPEALQA**	CLP 2499	**RLNPEALQA**
CLP 2503	**YPLFGLPFV**		
CLP 2509	**YVPYPTFNL**	CLP 2509	**YVPYPTFNL**
CLP 2511	**NLPPHFSAV**		
CLP 2518	**VMYALHMSC**		
CLP 2520	**GLHRNNAQV**	CLP 2520	**GLHRNNAQV**

D. Immune responses against BFA4 after immunization *in vivo*:

The pcDNA3.1/Zeo-BFA4 plasmid was used to immunize transgenic mice expressing a hybrid HLA-A*0201 α1α2 domain fused to a murine Kb α3 domain in C57BL/6 mice (A2-Kb mice). IFN-γ ELISPOT analysis using the groups of pooled peptides after DNA immunization and removal of activated spleen cells revealed a number of reactive BFA4 peptide groups. Some of these groups (especially group 7 and 8) also reacted strongly in human T-cell cultures suggesting that overlapping groups of peptides are recognized by human T cells and are naturally processed and presented on HLA-A2 after vaccination.

Vaccination experiments were also performed with the NYVAC-BFA4 and the MP76-18-BFA4 vectors in A2-Kb mice. Mice were immunized subcutaneously with 10-20μg of MP-76-18-BFA4 and 1-2 x 10^7 pfu vP2033 (NYVAC-BFA4) and boosted 28 days later with the

same amounts of each vector. Re-stimulation of spleen cells from the immunized mice with the pools of BFA4 peptides revealed induction of IFN-γ production in response to BFA4 peptide groups 2, 3, 4, 5, 7, 9, and 10 in ELISPOT assays. Thus, the BFA4 gene encoded in a CMV promoter driven eukaryotic plasmid, NYVAC, or a Semliki replicase-based DNA plasmid, were all capable of inducing T-cell responses against the BFA4 protein *in vivo*.

Example 2

BCY1 Tumor Antigen

The BCY1 gene was detected as a partial open reading frame (ORF) homologous to a nematode gene called "posterior-expressed maternal gene-3" (PEM-3) playing a role in posterior to anterior patterning in *Caenorhabtidis elegans* embryos. No previous involvement of this gene in cancer has been documented.

A. BCY1 and Amino Acid DNA Sequences

A partial DNA sequence was originally determined for BCY1. Primers, 9616SXC and 9617SXC, are derived from the BCY I partial DNA sequence and are designed to clone BCY I by RT-PCR from Calu 6 total RNA. The primers were designed such that the PCR product has BamHI sites at both ends and an ATG start codon and a Kozak sequence at the 5' end, as shown below:

9616SXC: 5' CAGTACGGATCCACCATGGCCGAGCTGCGCCTGAAGGGC 3'

9617SXC: 5' CCACGAGGATCCTTAGGAGAATATTCGGATGGCTTGCG 3'

The 1.2 Kb expected amplicon was obtained using ThermoScript RT-PCR System (Invitrogen) under optimized conditions. The PCR products from three separate RT-PCR's were digested with BamHI and respectively inserted in pcDNA3.1/Zeo(+). The resulting clones were MC50A6, MC50A8 and MC50A19 from the first RT-PCR; MC54.21 from the second RT-PCR and MC55.29; and, MC55.32 from the third RT-PCR (**Fig. 3**). The following primers were utilized in sequencing the clones:

9620MC: 5' TAATACGACTCACTATAGGG 3'
9621MC: 5' TAGAAGGCACAGTCGAGG 3'
9618MC: 5' GAAAACGACTTCCTGGCGGGGAG 3'

9619MC: 5' GCTCACCCAGGCGTGGGGCCTC 3'

DNA sequencing of all six clones indicated a consensus sequence, as shown in **Figs. 3A** and **B**, having the following differences from the original partial BCY1 sequence: a C to G substitution at position 1031 resulting in an amino acid change of Ala to Gly; a GC deletion at position 1032-1034 resulting in a Thr deletion; and, an A to G substitution at position 1177 resulting in an amino acid change of Thr to Ala. Clones MC50A8 and MC55.29 are identical to the consensus sequence. The amino acid sequence of BCY1 is shown in **Fig. 3B**.

B. Immunological reagents for BCY1 breast cancer antigen:

A library of 100 nonamer peptides spanning the BCY1 gene product was synthesized. The peptides were chosen based on their potential ability to bind to HLA-A*0201. **Table VIII** lists 100 nonamer peptide epitopes for HLA-A*0201 from the BCY1 protein tested (see below):

Table VIII

Peptide Designation	Sequence	Position in Protein
*CLP-2599	VPVPTSEHV	2
*CLP-2602	PTSEHVAEI	5
*CLP-2609	EIVGRQCKI	12
*CLP-2616	KIKALRAKT	19
*CLP-2618	KALRAKTNT	21
*CLP-2619	ALRAKTNTY	22
*CLP-2620	LRAKTNTYI	23
*CLP-2624	TN-TYIKTPV	27
*CLP-2627	YIKTPVRGE	30
*CLP-2630	TPVRGEEPV	33
*CLP-2633	RGEEPVFMV	36
*CLP-2640	MVTGRREDV	43
CLP-2641	VTGRREDVA	44
*CLP-2643	GRREDVATA	46
CLP-2647	DVATARREI	50
CLP-2648	VATARREII	51
*CLP-2650	TARREIISA	53
*CLP-2651	ARREIISAA	54
*CLP-2655	IISAAEHFS	58
*CLP-2656	ISAAEHFSM	59
CLP-2657	SAAEHFSMI	60
*CLP-2659	AEHFSMIRA	62
*CLP-2663	SMIRASRNK	66
CLP-2666	RASRNKSGA	69
*CLP-2670	NKSGAAFGV	73
*CLP-2673	GAAFGVAPA	76
*CLP-2674	AAFGVAPAL	77
*CLP-2677	GVAPALPGQ	80
*CLP-2678	VAPALPGQV	81
*CLP-2680	PALPGQVTI	83
*CLP-2681	ALPGQVTIR	84
*CLP-2682	LPGQVTIRV	85
CLP-2684	GQVTIRVRV	87
*CLP-2689	RVRVPYRVV	92
*CLP-2691	RVPYRVVGL	94
*CLP-2692	VPYRVVGLV	95
*CLP-2695	RVVGLVVGP	98
*CLP-2698	GLVVGPKGA	101
*CLP-2699	LVVGPKGAT	102
*CLP-2700	VVGPKGATI	103
*CLP-2710	RIQQQTNTY	113
*CLP-2711	IQQQTNTYI	114
*CLP-2712	QQQTNTYII	115
*CLP-2713	QQTNTYIIT	116
*CLP-2718	YIITPSRDR	121
CLP-2721	TPSRDRDPV	124
CLP-2724	RDRDPVFEI	127
CLP-2731	EITGAPGNV	134
CLP-2734	GAPGNVERA	137
CLP-2738	NVERAREEI	141
CLP-2744	EEIETHIAV	147
CLP-2746	IETHIAVRT	149

Table VIII (continued)

PEPTIDE DESIGNATION	SEQUENCE	POSITION IN PROTEIN
CLP-2749	HIAVRTGKI	152
CLP-2750	IAVRTGKIL	153
CLP-2756	KILEYNNEN	159
CLP-2760	YNNENDFLA	163
CLP-2762	NENDFLAGS	165
CLP-2766	FLAGSPDAA	169
CLP-2767	LAGSPDAAI	170
CLP-2774	AIDSRYSDA	177
CLP-2777	SRYSDAWRV	180
CLP-2785	VHQPGCKPL	188
CLP-2793	LSTFRQNSL	196
CLP-2801	LGCIGECGV	204
CLP-2807	CGVDSGFEA	210
CLP-2812	GFEAPRLDV	215
CLP-2817	RLDVYYGVA	220
CLP-2819	DVYYGVAET	222
CLP-2823	GVAETSPPL	226
CLP-2825	AETSPPLWA	228
CLP-2830	PLWAGQENA	233
CLP-2833	AGQENATPT	236
CLP-2835	QENATPTSV	238
CLP-2843	VLFSSASSS	246
CLP-2857	KARAGPPGA	260
CLP-2869	PATSAGPEL	272
CLP-2870	ATSAGPELA	273
CLP-2872	SAGPELAGL	275
CLP-2879	GLPRRPPGE	282
CLP-2887	EPLQGFSKL	290
CLP-2892	FSKLGGGGL	295
CLP-2894	KLGGGGLRS	297
CLP-2899	GLRSPGGGR	302
CLP-2909	CMVCFESEV	312
CLP-2910	MVCFESEVT	313
CLP-2911	VCFESEVTA	314
CLP-2913	FESEVTAAL	316
CLP-2916	EVTAALVPC	319
CLP-2917	VTAALVPCG	320
CLP-2920	ALVPCGHNL	323
CLP-2921	LVPCGHNLF	324
CLP-2922	VPCGHNLFC	325
CLP-2927	NLFCMECAV	330
CLP-2929	FCMECAVRI	332
CLP-2933	CAVRICERT	336
CLP-2936	RICERTDPE	339
CLP-2940	RTDPECPVC	343
CLP-2945	CPVCHITAT	348
CLP-2947	VCHITATQA	350
CLP-2950	ITATQAIRI	353

Table IX shows the groups of peptides used for immunological testing:

Peptide Group	Peptide Number	Peptid Sequence
1	CLP 2887	EPLQGFSKL
	CLP 2916	EVTAALVPC
	CLP 2945	CPVCHITAT
	CLP 2673	KIKALRAKT
	CLP 2699	IISAAEHFS
	CLP 2616	RASRNKSGA
	CLP 2655	GAAFGVAPA
	CLP 2731	LVVGPKGAT
	CLP 2734	EITGAPGNV
	CLP 2666	GAPGNVERA
2	CLP 2724	ALRAKTNTY
	CLP 2689	VATARREII
	CLP 2648	PALPGQVTI
	CLP 2680	ALPGQVTIR
	CLP 2619	RVRVPYRVV
	CLP 2681	RDRDPVFEI
	CLP 2689	RVRVPYRVV
	CLP 2947	HIAVRTGKI
	CLP 2762	NENDFLAGS
	CLP 2933	CAVRICERT
	CLP 2749	VCHITATQA
3	CLP 2647	GRREDVATA
	CLP 2677	DVATARREI
	CLP 2643	TARREIISA
	CLP 2785	GVAPALPGQ
	CLP 2917	RVVGLVVGP
	CLP 2695	VHQPGCKPL
	CLP 2650	PATSAGPEL
	CLP 2869	VTAALVPCG
4	CLP 2812	VPVPTSEHV
	CLP 2892	ARREIISAA
	CLP 2738	RIQQQTNTY
	CLP 2651	NVERAREEI
	CLP 2870	GFEAPRLDV
	CLP 2899	ATSAGPELA
	CLP 2710	FSKLGGGGL
	CLP 2599	GLRSPGGGR
5	CLP 2609	PTSEHVAEI
	CLP 2602	EIVGRQCKI
	CLP 2641	LRAKTNTYI
	CLP 2620	VTGRREDVA
	CLP 2940	SMIRASRNK
	CLP 2921	CMVCFESEV
	CLP 2936	LVPCGHNLF
	CLP 2663	NLFCMECAV
	CLP 2927	RICERTDPE
	CLP 2909	RTDPECPVC

Peptide Group	Peptide Number	Peptide Sequence
6	CLP 2766	MVTGRREDV
	CLP 2711	GLVVGPKGA
	CLP 2913	IQQQTNTYI
	CLP 2823	FLAGSPDAA
	CLP 2640	GVAETSPPL
	CLP 2698	FESEVTAAL
	CLP 2929	FCMECAVRI
7	CLP 2760	KALRAKTNT
	CLP 2633	RGEEPVFMV
	CLP 2700	SAAEHFSMI
	CLP 2835	AAFGVAPAL
	CLP 2618	VVGPKGATI
	CLP 2657	YNNENDFLA
	CLP 2674	LGCIGECGV
	CLP 2911	QENATPTSV
	CLP 2801	VCFESEVTA
8	CLP 2807	TNTYIKTPV
	CLP 2872	NKSGAAFGV
	CLP 2670	QQTNTYIIT
	CLP 2756	KILEYNNEN
	CLP 2825	CGVDSGFEA
	CLP 2843	AETSPPLWA
	CLP 2713	PLWAGQENA
	CLP 2624	VLFSSASSS
	CLP 2830	SAGPELAGL
9	CLP 2712	ISAAEHFSM
	CLP 2744	QQQTNTYII
	CLP 2774	EEIETHIAV
	CLP2819	IETHIAVRT
	CLP 2656	LAGSPDAAI
	CLP 2922	AIDSRYSDA
	CLP 2746	DVYYGVAET
	CLP 2767	VPCGHNLFC
	CLP 2950	ITATQAIRI
10	CLP 2793	TPVRGEEPV
	CLP 2777	AEHFSMIRA
	CLP 2910	VAPALPGQV
	CLP 2721	TPSRDRDPV
	CLP 2630	IAVRTGKIL
	CLP 2659	SRYSDAWRV
	CLP 2678	LSTFRQNSL
	CLP 2750	RLDVYYGVA
	CLP 2833	AGQENATPT
	CLP 2817	MVCFESEVT

C. Immune reactivity of BCY1 peptides and generation of human effector T cells:

The library of 100 peptides from BCY1 was separated into 10 groups of 7-10 peptides for immunological testing. Dissolved peptide pools were pulsed onto autologous HLA-A*0201 dendritic cells and used to activate autologous T-cell-enriched PBMC preparations. Activated T cells from each peptide-pool-stimulated culture were re-stimulated another 3 to 5 times using CD40L-activated autologous B-cells. IFN-γ ELISPOT analysis and assays for CTL killing of peptide-pulsed target cells was performed to demonstrate the immunogenicity of these epitopes from BCY1.

Human T cells demonstrated effector cell activity against a number of pools of peptides from the BCY1 protein, as shown by their ability to secrete IFN-γ in ELISPOT assays. These experiments were repeated after different rounds of APC stimulation resulting in the same reactive peptide groups. Peptide groups 1, 2, 3, 4, 5, 6, and 7 were found to be immunoreactive in these assays. Subsequently, these reactive peptide groups were de-convoluted in additional IFN-γ ELISPOT assays in which single peptides from each group were tested separately. This analysis revealed a number of individual strongly reactive peptides from the BCY1 protein recognized by human T cells. Many of these single peptides also induced CTL activity killing peptide-loaded human T2 lymphoma cell targets. **Table IX** lists these peptides.

While the present invention has been described in terms of the preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the appended claims cover all such equivalent variations that come within the scope of the invention as claimed.

SEQUENCE LISTING

SEQ ID NO.: 1: *BFA4 cDNA*

ATGGTCCGGAAAAAGAACCCCCCTCTGAGAAACGTTGCAAGTGAAGGCGAGGGCCAGATCCTGGAGCCTATAGGTACAGAAAGCAAGGTATCTGG
AAAGAACAAAGAATTCTCTGCAGATCAGATGTCAGAAAATACGGATCAGAGTGATGCTGCAGAACTAAATCATAAGGAGGAACATAGCTTGCATGTT
CAAGATCCATCTTCTAGCAGTAAGAAGGACTTGAAAAGCGCAGTTCTGAGTGAGAAGGCTGGCTTCAATTATGAAAGCCCCAGTAAGGGAGGAAACT
TTCCCTCCTTTCCGCATGATGAGGTGACAGACAGAAATATGTTGGCTTTCTCATTTCCAGCTGCTGGGGGAGTCTGTGAGCCCTTGAAGTCTCCGCA
AAGAGCAGAGGCAGATGACCCTCAAGATATGGCCTGCACCCCCTCAGGGGACTCACTGGAGACAAAGGAAGATCAGAAGATGTCACCAAAGGCTACA
GAGGAAACAGGGCAAGCACAGAGTGGTCAAGCCAATTGTCAAGGTTTGAGCCCAGTTTCAGTGGCCTCAAAAAACCCACAAGTGCCTTCAGATGGGG
GTGTAAGACTGAATAAATCCAAAACTGACTTACTGGTGAATGACAACCCAGACCCGGCACCTCTGTCTCCAGAGCTTCAGGACTTTAAATGCAATAT
CTGTGGATATGGTTACTACGGCAACGACCCCACAGATCTGATTAAGCACTTCCGAAAGTATCACTTAGGACTGCATAACCGCACCAGGCAAGATGCT
GAGCTGGACAGCAAAATCTTGGCCCTTCATAACATGGTGCAGTTCAGCCATTCCAAAGACTTCCAGAAGGTCAACCGTTCTGTGTTTTCTGGTGTGC
TGCAGGACATCAATTCTTCAAGGCCTGTTTTACTAAATGGGACCTATGATGTGCAGGTGACTTCAGGTGGAACATTCATTGGCATTGGACGGAAAAC
ACCAGATTGCCAAGGGAACACCAAGTATTTCCGCTGTAAATTCTGCAATTTCACTTATATGGGCAACTCATCCACCGAATTAGAACAACATTTTCTT
CAGACTCACCCAAACAAAATAAAAGCTTCTCTCCCCTCCTCTGAGGTTGCAAAACCTTCAGAGAAAAACTCTAACAAGTCCATCCCTGCACTTCAAT
CCAGTGATTCTGGAGACTTGGGAAAATGGCAGGACAAGATAACAGTCAAAGCAGGAGATGACACTCCTGTTGGGTACTCAGTGCCCATAAAGCCCCT
CGATTCCTCTAGACAAAATGGTACAGAGGCCACCAGTTACTACTGGTGTAAATTTTGTAGTTTCAGCTGTGAGTCATCTAGCTCACTTAAACTGCTA
GAACATTATGGCAAGCAGCACGGAGCAGTGCAGTCAGGCGGCCTTAATCCAGAGTTAAATGATAAGCTTTCCAGGGGCTCTGTCATTAATCAGAATG
ATCTAGCCAAAAGTTCAGAAGGAGAGACAATGACCAAGACAGACAAGAGCTCGAGTGGGGCTAAAAAGAAGGACTTCTCCAGCAAGGGAGCCGAGGA
TAATATGGTAACGAGCTATAATTGTCAGTTCTGTGACTTCCGATATTCCAAAAGCCATGGCCCTGATGTAATTGTAGTGGGGCCACTTCTCCGTCAT
TATCAACAGCTCCATAACATTCACAAGTGTACCATTAAACACTGTCCATTCTGTCCCAGAGGACTTTGCAGCCCAGAAAAGCACCTTGGAGAAATTA
CTTATCCGTTTGCTTGTAGAAAAAGTAATTGTTCCCACTGTGCACTCTTGCTTCTGCACTTGTCTCCTGGGGCGGCTGGAAGCTCGCGAGTCAAACA
TCAGTGCCATCAGTGTTCATTCACCACCCCTGACGTAGATGTACTCCTCTTTCACTATGAAAGTGTGCATGAGTCCCAAGCATCGGATGTCAAACAA
GAAGCAAATCACCTGCAAGGATCGGATGGGCAGCAGTCTGTCAAGGAAAGCAAAGAACACTCATGTACCAAATGTGATTTTATTACCCAAGTGGAAG
AAGAGATTTCCCGACACTACAGGAGAGCACACAGCTGCTACAAATGCCGTCAGTGCAGTTTTACAGCTGCCGATACTCAGTCACTACTGGAGCACTT
CAACACTGTTCACTGCCAGGAACAGGACATCACTACAGCCAACGGCGAAGAGGACGGTCATGCCATATCCACCATCAAAGAGGAGCCCAAAATTGAC
TTCAGGGTCTACAATCTGCTAACTCCAGACTCTAAAATGGGAGAGCCAGTTTCTGAGAGTGTGGTGAAGAGAGAGAAGCTGGAAGAGAAGGACGGGC
TCAAAGAGAAAGTTTGGACCGAGAGTTCCAGTGATGACCTTCGCAATGTGACTTGGAGAGGGGCAGACATCCTGCGGGGGAGTCCGTCATACACCCA
AGCAAGCCTGGGGCTGCTGACGCCTGTGTCTGGCACCCAAGAGCAGACAAAGACTCTAAGGGATAGTCCCAATGTGGAGGCCGCCCATCTGGCGCGA
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CGGGAGAAAACAAGTCCAAGGATGAATCCCAGTCCCTGTTACGGAGGCGTAGAGGCTCCGGTGTTTTTTGTGCCAATTGCCTGACCACAAAGACCTC
TCTCTGGCGAAAGAATGCAAATGGCGGATATGTATGCAACGCGTGTGGCCTCTACCAGAAGCTTCACTCGACTCCCAGGCCTTTAAACATCATTAAA
CAAAACAACGGTGAGCAGATTATTAGGAGGAGAACAAGAAAGCGCCTTAACCCAGAGGCACTTCAGGCTGAGCAGCTCAACAAACAGCAGAGGGGCA
GCAATGAGGAGCAAGTCAATGGAAGCCCGTTAGAGAGGAGGTCAGAAGATCATCTAACTGAAAGTCACCAGAGAGAAATTCCACTCCCCAGCCTAAG
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TGTACATAATGACTTCCAGAGTGAAGCTGATTGGCTGCGGTTCTGGAGTAAATATAAGCTCTCCGTTCCTGGGAATCCGCACTACTTGAGTCACGTG
CCTGGCCTACCAAATCCTTGCCAAAACTATGTGCCTTATCCCACCTTCAATCTGCCTCCTCATTTTTCAGCTGTTGGATCAGACAATGACATTCCTC
TAGATTTGGCGATCAAGCATTCCAGACCTGGGCCAACTGCAAACGGTGCCTCCAAGGAGAAAACGAAGGCACCACCAAATGTAAAAAATGAAGGTCC
CTTGAATGTAGTAAAAACAGAGAAAGTTGATAGAAGTACTCAAGATGAACTTTCAACAAAATGTGTGCACTGTGGCATTGTCTTTCTGGATGAAGTG
ATGTATGCTTTGCATATGAGTTGCCATGGTGACAGTGGACCTTTCCAGTGCAGCATATGCCAGCATCTTTGCACGGACAAATATGACTTCACAACAC
ATATCCAGAGGGGCCTGCATAGGAACAATGCACAAGTGGAAAAAAATGGAAAACCTAAAGAGTAA

SEQ ID NO.: 2: BFA4 Amino Acid Sequence

MVRKKNPPLRNVASEGEGQILEPIGTESKVSGKNKEFSADQMSENTDQSDAAELNHKEEHSLHVQDPSSS
SKKDLKSAVLSEKAGFNYESPSKGGNFPSFPHDEVTDRNMLAFSFPAAGGVCEPLKSPQRAEADDPQDMA
CTPSGDSLETKEDQKMSPKATEETGQAQSGQANCQGLSPVSVASKNPQVPSDGGVRLNKSKTDLLVNDNP
DPAPLSPELQDFKCNICGYGYYGNDPTDLIKHFRKYHLGLHNRTRQDAELDSKILALHNMVQFSHSKDFQ
KVNRSVFSGVLQDINSSRPVLLNGTYDVQVTSGGTFIGIGRKTPDCQGNTKYFRCKFCNFTYMGNSSTEL
EQHFLQTHPNKIKASLPSSEVAKPSEKNSNKSIPALQSSDSGDLGKWQDKITVKAGDDTPVGYSVPIKPL
DSSRQNGTEATSYYWCKFCSFSCESSSSLKLLEHYGKQHGAVQSGGLNPELNDKLSRGSVINQNDLAKSS
EGETMTKTDKSSSGAKKKDFSSKGAEDNMVTSYNCQFCDFRYSKSHGPDVIVVGPLLRHYQQLHNIHKCT
IKHCPFCPRGLCSPEKHLGEITYPFACRKSNCSHCALLLLHLSPGAAGSSRVKHQCHQCSFTTPDVDVLL
FHYESVHESQASDVKQEANHLQGSDGQQSVKESKEHSCTKCDFITQVEEEISRHYRRAHSCYKCRQCSFT
AADTQSLLEHFNTVHCQEQDITTANGEEDGHAISTIKEEPKIDFRVYNLLTPDSKMGEPVSESVVKREKL
EEKDGLKEKVWTESSSDDLRNVTWRGADILRGSPSYTQASLGLLTPVSGTQEQTKTLRDSPNVEAAHLAR
PIYGLAVETKGFLQGAPAGGEKSGALPQQYPASGENKSKDESQSLLRRRRGSGVFCANCLTTKTSLWRKN
ANGGYVCNACGLYQKLHSTPRPLNIIKQNNGEQIIRRRTRKRLNPEALQAEQLNKQQRGSNEEQVNGSPL
ERRSEDHLTESHQREIPLPSLSKYEAQGSLTKSHSAQQPVLVSQTLDIHKRMQPLHIQIKSPQESTGDPG
NSSSVSEGKGSSERGSPIEKYMRPAKHPNYSPPGSPIEKYQYPLFGLPFVHNDFQSEADWLRFWSKYKLS
VPGNPHYLSHVPGLPNPCQNYVPYPTFNLPPHFSAVGSDNDIPLDLAIKHSRPGPTANGASKEKTKAPPN
VKNEGPLNVVKTEKVDRSTQDELSTKCVHCGIVFLDEVMYALHMSCHGDSGPFQCSICQHLCTDKYDFTT
HIQRGLHRNNAQVEKNGKPKE

SEQ ID NO.: 3: *BCY1 cDNA Sequence*

ATGGCCGAGCTGCGCCTGAAGGGCAGCAGCAACACCACGGAGTGTGTTCCCGTGCCCACCTCCGAGCACGTGGCCGA
GATCGTGGGCAGGCAAGGCTGCAAGATTAAGGCCTTGAGGGCCAAGACCAACACCTACATCAAGACACCGGTGAGGG
GCGAGGAACCAGTGTTCATGGTGACAGGGCGACGGGAGGACGTGGCCACAGCCCGGCGGGAAATCATCTCAGCAGCG
GAGCACTTCTCCATGATCCGTGCCTCCCGCAACAAGTCAGGCGCCGCCTTTGGTGTGGCTCCTGCTCTGCCCGGCCA
GGTGACCATCCGTGTGCGGGTGCCCTACCGCGTGGTGGGGCTGGTGGTGGGCCCCAAAGGGGCAACCATCAAGCGCA
TCCAGCAGCAAACCAACACATACATTATCACACCAAGCCGTGACCGCGACCCCGTGTTCGAGATCACGGGTGCCCCA
GGCAACGTGGAGCGTGCGCGCGAGGAGATCGAGACGCACATCGCGGTGCGCACTGGCAAGATCCTCGAGTACAACAA
TGAAAACGACTTCCTGGCGGGGAGCCCCGACGCAGCAATCGATAGCCGCTACTCCGACGCCTGGCGGGTGCACCAGC
CCGGCTGCAAGCCCCTCTCCACCTTCCGGCAGAACAGCCTGGGCTGCATCGGCGAGTGCGGAGTGGACTCTGGCTTT
GAGGCCCCACGCCTGGGTGAGCAGGGCGGGGACTTTGGCTACGGCGGGTACCTCTTTCCGGGCTATGGCGTGGGCAA
GCAGGATGTGTACTACGGCGTGGCCGAGACTAGCCCCCCGCTGTGGGCGGGCCAGGAGAACGCCACGCCCACCTCCG
TGCTCTTCTCCTCTGCCTCCTCCTCCTCCTCTTCCGCCAAGGCCCGCGCTGGGCCCCCGGGCGCACACCGCTCC
CCTGCCACTTCCGCGGGACCCGAGCTGGCCGGACTCCCGAGGCGCCCCCCGGGAGAGCCGCTCCAGGGCTTCTCTAA
ACTTGGTGGGGGCGGCCTGCGGAGCCCCGGCGGCGGGCGGGATTGCATGGTCTGCTTTGAGAGCGAAGTGACTGCCG
CCCTTGTGCCCTGCGGACACAACCTGTTCTGCATGGAGTGTGCAGTACGCATCTGCGAGAGGACGGACCCAGAGTGT
CCCGTCTGCCACATCACAGCCGCGCAAGCCATCCGAATATTCTCCTAA

SEQ ID NO.: 4: *BCY1 Amino Acid Sequence*

MAELRLKGSS NTTECVPVPT SEHVAEIVGR QGCKIKALRA KTNTYIKTPV RGEEPVFMVT GRREDVATAR
REIISAAEHF SMIRASRNKS GAAFGVAPAL PGQVTIRVRV PYRVVGLVVG PKGATIKRIQ QQTNTYIITP
SRDRDPVFEI TGAPGNVERA REEIETHIAV RTGKILEYNN ENDFLAGSPD AAIDSRYSDA WRVHQPGCKP
LSTFRQNSLG CIGECGVDSG FEAPRLGEQG GDFGYGGYLF PGYGVGKQDV YYGVAETSPP LWAGQENATP
TSVLFSSASS SSSSSAKARA GPPGAHRSPA TSAGPELAGL PRRPPGEPLQ GFSKLGGGGL RSPGGGRDCM
VCFESEVTAA LVPCGHNLFC MECAVRICER TDPECPVCHI TAAQAIRIFS